Response of Genotypes, explants and culture media for *in vitro* regeneration in *Trifolium alexandrinum* and allied species

THESIS

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By

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Dedicated

To

The Loving Memory of

MY FATHER

डा॰ प्रेम शंकर पाठक

निदेशक

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Director

PS/8-1/01

April 21. 2001

The Vice Chancellor.
Bundelkhand University.
Jhansi

Sub: **Submission of Ph.D. Thesis**

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Sir.

I am forwarding herewith the thesis entitled "Response of genotypes explants and culture media for *in vitro* regeneration in *Trifolium alexandrinum* and allied species" by Aparna Tiwari for the degree of Doctor of Philosophy in Botany. Bundelkhand University. Jhansi. The work has been carried out at Indian Grassland and Fodder Research Institute. Jhansi under the supervision of Dr. A.K. Roy.

Thanking you.

Yours faithfully.

(P.S. Pathak)

CERTIFICATE

It is certified that this thesis entitled "Response of genotypes, explants and culture media for *in vitro* regeneration in *Trifolium alexandrinum* and allied species." is an original piece of work done by Aparna Tiwari, M.Sc. (Botany) under my supervision and guidance for the degree of Doctor of Philosophy in Botany, Bundelkhand University, Jhansi.

I, further certify that

- It embodies the original work of candidate himself
- It is up to the required standard both in respect of its contents and literary presentation for being referred to the examiners.
- The candidate has worked under me for the required period at Indian Grassland and Fodder Research Institute Jhansi.
- The candidate has put in the required attendance in the department during the period (December, 1997 to till date).

A. K. Roy 21/4/2001
(Supervisor)

DECLARATION

I hereby declare that the thesis entitled "Response of genotypes, explants and culture media for *in vitro* regeneration in *Trifolium alexandrinum* and allied species." being submitted for the degree of Doctor of Philosophy in Botany, Bundelkhand University, Jhansi (UP) is an original piece of research work done by me under the supervision of Dr. A. K. Roy, IGFRI, Jhansi and to the best of my knowledge, any part or whole of this thesis has not been submitted for a degree or any other qualification of any university or examining body in India / elsewhere.

Tiwari 21/04/01
(APARNA TIWARI)

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APARNA TIWARI.

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INTRODUCTION

1. INTRODUCTION

The genus *Trifolium,* commonly called clovers, comprises of 237-300 species (Taylor, 1980; Chen and Gibson, 1971; Zohary and Heller, 1984) out of which 25 are agriculturally important as cultivated forage and pasture crops (Lange and Schifino-Wittmann, 2000). The species include both annual and perennial types and are distributed over temperate to sub-temperate and tropical to sub-tropical climate.

The center of diversity of this genus lies in the Mediterranean belt. Out of 237 species of the genus, 110 species are found in the Mediterranean region, which has been identified as one of the main centers of distribution and also a center of domestication and breeding (Zohary and Heller, 1984) Another center of distribution with lesser number of species could be Californian region which is considered as a primary center of speciation of the genus. It has been suggested that some of the species native to the NW part of America migrated to Asia and then spread to the Mediterranean area where they created a highly diversified speciation center (Zohary, 1972).

The common and important perennial pasture species of the genus are *T. repens* (white clover), *T. pratense* (red clover), *T. hybridum* (alsike clover), *T. ambiguum* (caucasian clover). These species are widely distributed in the temperate and sub-temperate regions of the world. The most common annual species are *T. alexandrinum* (Egyptian clover or Berseem), *T. resupinatum* (Persian clover or shaftal) and *T. subterraneum* (subterranean clover), cultivated as winter annuals in the subtropical region

T. alexandrinum commonly called as Egyptian clover or Berseem is believed to have originated in Egypt and / or other Mediterranean countries. Its center of diversity lies in Mediterranean zone such as Egypt and Turkey. Berseem clover is an important cool season annual forage crop of the Mediterranean region, near east and India (Knight, 1985 ; Fairbrother, 1996).

Berseem cultivar 'Mescavi' or 'Meskawi' was introduced in India in the year 1903 (Whyte, 1978). It has become the most important winter season legume fodder in central, north and north west India. It is widely adapted as a cultivated, nutritive green fodder for the dairy animals and is cultivated in about 2 million hactare area with an average national productivity of 85 t /ha green fodder. Certain features like multicut nature (4-6 cuts/season), long duration of green fodder availability (November to April), high yield (85 t/ha), good quality (20% crude protein), good digestibility (70% IVDMD) and palatability have made it highly acceptable among farmers.

Considering these good attributes, coupled with wide adaptation in tropical and sub tropical parts of the country the crop has attracted serious attention of research workers for its further genetic improvement. The present day cultivars lack the source of resistance for various biotic and abiotic stresses such as root and stem rot diseases and tolerance to salt/ very high or low temperature.

Genetic improvement efforts through conventional breeding and biotechnological techniques

Since its introduction in India in early part of 20th century, efforts have been carried out in various universities and research Institution in India for its genetic improvement which have resulted in some initial success, however no major breakthrough has yet been achieved. Lack of genetic variability has been identified as one of the major bottleneck in its genetic improvement efforts. A brief account of various conventional breeding and biotechnological efforts carried out in the past and their major outcome is given below.

Introduction- Berseem or *Trifolium alexandrinum* was introduced in India in 1903 (Whyte, 1978). Initially two biotypes 'Fahli' and 'Mescavi' were introduced . Mescavi became popular and was well adopted by farmers as winter annual crop in northern, central and north western part of the country. However, in the last few decades only a few exotic lines could be introduced which have not contributed significantly to the genetic improvement programme (Malaviya & Rao, 1997).

Selection- Several attempts have been made for identifying better varieties through mass selection, recurrent selection, restricted recurrent phenotypic selection methods (Radwan *et al.,* 1972; Burton, 1979; Bakheit, 1989; Mahdy, 1988). These efforts have resulted in the development of present day cultivars such as JHB 146, Wardan, BL 1, BL 10 *etc.* These cultivars show only marginal superiority for forage yield. The selective breeding for higher yield has resulted   in narrowing the genetic base of populations, thereby most of the cultivars lack the genes for various desirable attributes such as tolerance to biotic and abiotic stress, photoperiod insensitivity, higher quality *etc.*

Mutation- Mutation breeding efforts by several workers (Shukla, 1986; Shukla and Tripathi, 1983; Jatsara, 1981; Jatsara *et al.* 1980) have met with very little success and have not resulted in any significant genetic variability of stable nature.

Polyploidization- A major breakthrough in berseem improvement was achieved through polyploidy in early sixties and a tetraploid line named as 'Pusa giant' was developed. The line performed better than the diploid counterpart for green fodder yield (Sikka *et al.,* 1959; Mehta and Swaminathan, 1957; Mehta *et al.,* 1964). However, the variety underwent depolyploidization and could not be stabilized (Singh *et al.,* 1988).   Subsequent efforts were carried out at IGFRI and a few more tetraploid lines have been created (Roy, 1995; Roy *et al.,* 1998).

Interspecific   hybridization   : Interspecific   hybridization   involving   *T. alexandrinum* has failed through conventional techniques (Anonymous, 1990, 1991). Various studies have indicated the existence of crossability barrier at post zygotic stage resulting in arrest of embryo development and endosperm disintegration. Embryo development in various interspecific crosses in *Trifolium* is reported to stop after a few days of pollination. The hybrid embryo grows up to heart shaped stage in *T. ambiguum x T. repens* (Williams and White, 1976); up to globular stage in *T. semiplosum x T. repens* (White and Williams, 1976); Slower mitotic rate after 4-5 days was reported in *T. repens x T. medium* hybrid embryo (Kazimierska, 1978). These studies suggest that the action of deleterious genes are initiated at the time of fertilization or shortly thereafter.

Several reports indicate endosperm disintegration in the interspecific crosses, thereby depriving nutrition to the embryos. Williams and White (1976) reported that in *T. ambiguum x T. repens* crosses the endosperm development never proceeded beyond the 128 nucleate stage, whereas selfed *T. ambiguum* seed had several thousand cells in the endosperm. To overcome these post-fertilization barriers *in vitro* embryo rescue has been successfully employed in various cases resulting in production of several interspecific hybrids. The procedure involves rescuing the hybrid embryo a few days after fertilization and its subsequent development in aseptic *in vitro* nutrient media. This enables the embryo to express its latent capacity to develop and produce a plant.

Successful interspecific hybrids involving *T. repens, T. pratense, T. alpestre, T. sarosiense, T. rubens, T. incarnatum* in various combinations have been reported (Evans, 1962 a, b; Phillips *et al.*, 1982; Collins *et al.*, 1981). However, reports are not available about interspecific crosses involving the tropical species *T. alexandrinum* which may have specific requirement for regeneration.

Somaclonal variation- Creation of diverse plant types by exploiting somaclonal variation has been attempted in different species of *Trifolium viz. T. repens, T. pratense, T. subterraneum, T. rubens.* However, in *T. alexandrinum* only a few sporadic reports of *in vitro* regeneration are available (Mokhtarzadeh and Constantin, 1978; Barakat, 1990).

Considering above mentioned points the conclusion can be drawn that the genetic diversity in the indigenous and exotic berseem genotypes is very narrow. Its poor performance in sub temperate and temperate zone, alkaline/saline soils as well as high sensitivity to temperature and photoperiod restricts this crop to only north central plains from October to April. Furthermore, prevalence of disease like root rot and stem rot is causing severe damage to this crop in north western parts such as Punjab and Haryana (Bhaskar and Ahmad, 1990). Various efforts through conventional techniques carried out during last 4 decades have so far not been able to produce any significant result. The efforts have reached at a road block where biotechnological efforts need to be considered for a break through.

The plausible approach, therefore, to broaden the genetic base of Berseem is through different biotechnological techniques such as *in vitro* regeneration, somaclonal variation, somatic embryogenesis, genetic transformation *etc.* Transfer of alien genes from wild taxa through somatic hybridization together with *in vitro* embryo rescue following interspecific hybridization may help in broadening the genetic base.

To achieve the above mentioned objectives the primary requirement is to develop suitable protocol for *in vitro* regeneration and successful establishment of regenerants in field conditions. The characterization of regenerants at various stages of development will give a picture of the variability.

Different species and genotypes may have different nutritional requirements for *in vitro* development, the study therefore should be planned to carry out using different explants, genotypes and media. The protocol developed for *in vitro* regeneration in different species can be exploited in the long run for various biotechnological tools such as somatic hybridization, somaclonal variation, genetic transformations *etc.*

The proposed programme was therefore carried out with the long term objective of creation of genetic diversity in different *Trifolium* sp. particularly *T. alexandrinum.* In the present study the efforts were made

- to develop suitable protocol for *in vitro* regeneration in *Trifolium alexandrinum* and other *Trifolium* species.

- to induce genetic variability through somaclonal variation

- to characterize callus/regenerant at various stages of growth.

REVIEW

2. Review of Literature

Plant tissue, cell and protoplast cultures have become useful tools for crop improvement, especially as supplementary means of inducing variability. Tissue culture instability and somaclonal variations are ubiquitous and offer possible benefits as adjuncts to plant improvement (Scowcroft, 1984). The ability to regenerate large number of plants from cultured tissue is important for the successful application of this technology to crop improvement.

The ability to regenerate whole plants is a vital objective of *in vitro* tissue culture, and is necessary for the application of molecular and somatic genetics to crop improvement. Plant regeneration from various experimental materials is the most important component of the tissue culture system, and a critical element in translating the laboratory results into practical application (Morginski and Kartha, 1984).

2.1. Tissue culture media components

A significant factor for the success in tissue culture is the choice of nutritional components and growth regulators. The successful establishment and growth of plant cells *in vitro* generally is determined by the nature of the explant and the composition of the nutrient media. In the last three or four decades a large number of reports have appeared on modification of about two dozen basic constituents.

Nutritional requirements for optimal growth of a tissue *in vitro* may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962). When starting with a new system it is essential to work out a medium that would fulfil the specific requirements of that tissue.

Selection of a culture medium for *in vitro* studies would seem baffling in view of the several well established media and their numerous modifications. Macro and microelement composition of most media is more or less common to all (differing only in their concentrations and Nitrogen source) and any proposed

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change pertains mostly to exogenous supply of vitamins, auxins, cytokinins and other growth factors. The choice of a nutrient media is based more on its mineral composition than on its other components. Suitable alterations in the medium composition are warranted depending on the objective of the experiment. A culture medium suitable for callus growth may not be suitable for maintenance of the callus or regeneration or induction of somatic embryos. For example, the high salt media, which are excellent for supporting callus growth and morphogenesis, have not proved very suitable for the growth of excised roots, anthers and other floral organs,. Whereas, White's medium is very good for the culture of excised roots (Street, 1967); Nitsch's (1951) formulation is good for the culture of excised floral organs and the Nitsch and Nitsch (1969) medium for obtaining haploid tissues or embryoids from cultured anthers.

Media constituents

A nutrient medium usually consists of inorganic salts (major and minor) a carbon source, some vitamins and growth regulators. The components of all growth media can be grouped under organic and inorganic constituents.

2.1.1 Inorganic nutrients

The mineral nutrients required for successful growth of plant have been divided by Clarkson and Hanson (1980) into two major groups. (i) Elements like N,P,S that are covalently bonded in carbon components and are vital constituents of the macromolecules, DNA, RNA and protein. (ii) other elements like K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Mo, B *etc.* that participate in a variety of often overlapping functions including regulation of osmotic and electrical gradients, protein conformation and oxidation- reduction reactions of metalloprotein.

Based on the quantities of requirement media components can be grouped into macro and micro elements

(A) **Macroelements** : Out of various elements essential for plant growth six elements (N, P, S, Ca, K, Mg) are required in comparatively large quantities and are therefore termed macro or major elements.

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(B) Microelements :   The microelements usually included in plant tissue culture media are Fe, Mn, B, Zn, Mo, Cu, I and Co.

A brief account of role and mode of supplementation of various inorganic elements in the media is as under :

Inorganic Components

Sulphur :   Sulphur is primarily supplied as sulphate (SO_4^-) and is utilized for protein synthesis *via* sulphate respiration as soluble cysteine (99.9%) and a smaller portion as soluble methionine (Giovanelli *et al.*., 1980). Sulphur is also present in such substances as glutathione, believed to be concerned with oxidation reduction reaction in plants. The sulphur requirements of a culture vary depending on the object (0.5 to 10 mM).

Phosphorus : Phosphorus is a structural component of the nucleic acids, DNA and RNA.   As a part of the fatty substances, the phospholipids are an essential structural component of the cell membrane. Phosphorus is also involved in all energy-transfer processes in the cell and compounds such as ATP are composed of thee phosphates coupled to a complicated ring structure. Phosphorus is commonly added as PO_4^{3-} at concentrations of 1.1-1.25 mM (Murashige and Skoog, 1962). Due to rapid uptake and interactions with other components (Fe, K, Saccharose), deficiencies may rapidly arise in a medium. In addition, its uptake in influenced by the supply of other elements. For example, Boron deficiency induces a reduction in the phosphorus uptake capacity in *Daucus carota* cultures (Goldbach, 1985).

Nitrogen: Among all the mineral nutrients, the form of Nitrogen (Oxidized or reduced, organic or inorganic), probably is responsible for the most pronounced effect on growth and differentiation of cultured tissues. Most standard media offer nitrogen as NH_4^+ and NO_3^-. Individual cultures (*Cannabis sativa, Ipomoea, Daucus carota*) prefer NH_4^+ under certain conditions. Utilization of NO_3^- requires functioning nitrate reductase, the presence of which has by now been demonstrated in numerous callus and suspension cultures (Bray, 1983). Nitrogen supplied in the

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form of nitrate (NO_3^-) is readily absorbed by most plants, but the form in which such nitrogen is incorporated into the plant, is highly reduced such as in the amino-group ($-NH_2$). In a few cases, other sources of N may replace NO_3^- or NH_4^+ as nitrogen source, or they may augment the existing supply.

Magnesium, Potassium, Calcium : The cations Mg, K and Ca play an essential role in cell metabolism. Mg^{++} is one of the essential factors in translation. Its functions include action as co-factor (e.g. glutamine synthase) and activator of various enzymes. Mg is an essential constituents of the chlorophyll molecule.

The Ca^{++} is also essential for deposition of phospholipids and proteins on or within plasma membranes. Its importance is further demonstrated by the efforts of cells to maintain their intracellular concentration at 10^{-6} to 10^{-8} M even against a concentration gradient using specific Ca^{++} pumps and Ca^{++} binding proteins (calmodulin) located in the cytoplasm and/or individual organelles. Calcium is also a constituent of the middle lamella of the cell wall.

Potassium is supplied at concentration 20mM or higher as the nitrate or chloride, and chloride ions are important in that they stimulate the production of necessary enzymes.

Microelements :

The microelements Fe, Mn, Zn, Cu, Mo, I, B and Co act as co-factors and as inducer of enzyme synthesis. Boron is essential for membrane function, permeability and integrity thereby influencing membrane fixed processes, membrane potential and phytohormone metabolism. Lack of iron results in increased contents of DNA and free amino acids, as well as a reduced RNA content. In order to maintain a minimum supply of Fe it is therefore usually added in complexes with EDTA or sequestrin. This also facilitates uptake over a broad pH range, which varies depending on the content of phosphate, NO_3 and NH_4^+ in the medium.

These eight elements take part in catalytic processes going on in the cell. Copper is a part of certain oxidative oxidase which serve to oxidise phenolic

substances. Iron functions as a respiratory electron carrier through compounds such as cytochromes and the oxidative enzymes, peroxidase and catalase. The exact role of Boron in cell metabolism is rather obscure, though implicated in sugar transport in speeding up the rate of sugar movement in the plant.

II. Organic nutrients

Three groups of organic nutrients are required in tissue cultures,

1.   Carbohydrates
2.   Vitamins
3.   Plant growth regulators

1. **Carbohydrates** - A variety of carbon sources is used in tissue culture media. Cell cultures are usually cultivated heterotrophically and, in most cases, carbon must be added in the form of carbohydrate. The disaccharide, sucrose beings the most common. Sucrose is a necessary component in the culture media as most tissue cultures are not autotrophic. Sucrose as a precursor in reduced concentrations, serves to minimize the quantum of alcohol production. It also acts as the main osmoticum, besides being an energy source.

While sucrose is incorporated in the medium usually in the optimal range of 2-4% (W/V) for growth and morphogenesis of most tissues, there are reports that organogenesis in individual cases may require altered levels of sucrose (Narayanswamy, 1994). Some tissues may prefer specific sugar such as maltose, glucose and fructose or sorbitol.

2. **Vitamins** - The medium requires to be supplemented by one or more vitamins of the water soluble B-complex for healthy growth of tissues in culture. They are apparently synthesized in sub-optimal quantities by callus tissues. Vitamins play a catalytic role in cell metabolism apart from being a factor in accessory food supply, but their requirements vary from species to species.

3. **Plant growth regulators** : In addition to the nutrients, it is generally necessary to add hormones. The supplementation of phytohormones are necessary to trigger cell division in a tissue explant and sustained growth of the cultures. However,

the requirement of these hormone varies considerably with the tissue, and it is believed that it depends on their endogenous levels.

The growth process of a culture is decisively affected by the ratio of auxins to cytokinin. It is widely believed that plants regulate the levels of active auxins and cytokinins by both synthesis and conjugation. In general, higher auxin and low cytokinin concentrations stimulate cell division, while low auxin and high cytokinin concentrations stimulate cell growth. However, an excessive supply of gibberellic acid and phenolic compounds cancels this balance.

There are five known classes of growth substances Auxins, Cytokinins, Gibberlins, Ethylene, Abscisic Acid (Narayanswamy, 1994). However, only two (Auxins and Cytokinins) are widely used in the culture media.

Auxins - Growth substances based on the indole nucleus are referred to as auxins. Auxins may initiate or promote cell division from tissues cultured *in vitro*, can stimulate shoot growth, control vascular system differentiation, regulate apical dominance, delay senescence, promote flowering. The growth and viability of cultures are usually ensured by adding synthetic (2,4-D, NAA) or naturally occurring (IAA) auxins.

Cytokinins - Cytokinins are 6- substituted purine compounds. They are now fully recognised as one of the major groups of endogenous plant hormones. In tissue culture media, cytokinins are incorporated mainly for cell division and differentiation of adventitious shoots from callus (Bhojwani and Rajdan, 1983)..

Cytokinin is arbitrarily defined in terms of its capacity to promote *in-vitro* cell division and growth of callus tissues in the same manner as kinetin stimulates growth of cultured cells. In addition to the natural cytokinin, such as, Zeatin (r-hydroxy methyl-adenine) and 2-ip (isopentyl-r,r-di-methyl-allyl), synthetic products such as kinetin (6-furfurylaniinopurine), 6-BAP (N6-benzylamino purine) are also used.

Culture media pH : The pH of the medium greatly influences the uptake of ingredients, solubility of salts and gelling efficiency of agar. Although the pH of

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the medium is altered during culture, an initial pH is selected before autoclaving. The pH is adjusted using NaOH or HCl. A pH of 5.6-5.8 has been found suitable for maintaining all the salts in a near buffered form.

Solidification of Medium : Because of improved oxygen supply and the developing chemical gradients, cultures on solid media are often preferred to liquid cultures. For this purpose, substances with a strong gelling capacity are added to the liquid medium. These reversibly bind water and thus ensure the humidity of the medium desired for culturing, depending on the concentration. An inert natural plant product agar is used very commonly in tissue culture experiments as solidifying agent.

2.2. *In vitro* plant regeneration

2.2.1. Callus induction

Aseptic culture of the damaged region on a defined medium can cause cell division which can be stipulated and induced to continue indefinitely through the exogenous influence of the chemical constitution of the culture medium. The result is continually dividing mass of poorly differentiated and disorganised plant cell aggregates termed a callus.

In morphological terms it can vary extensively, ranging from being very hard/compact where the cells have extensive and strong cell to cell contact, to being friable where the callus consists of small, disintegrating aggregates of poorly associated cells and has a rather crumbly or creamy appearance. Friable callus is generally most sought after as it is usually the fastest growing and most uniform type and is best suitable for the initiation of cell suspension cultures. Callus morphology is often dependant on explant but can be altered by the modification / supplementation of the growth substance to the culture medium.

Due to its size and nature, callus cultures have as inherent degree of heterogeneity. As there is a unidirectional supply of nutrients (from the medium below) and gases and light (predominantly from above), chemical and physical

gradients will be present within the callus mass. While, in some instances, this heterogeneity is a disadvantage (e.g. in the production of uniform biomass) it may also be an important factor influencing the developmental response of the callus in plant regeneration (Hall, 1991).

Influence of cell origins and genotype on callus type : In normal practice callus cultures are established from multicellular pieces of vegetative tissues. These fragments may be relatively homogeneous with respect to a particular cell type, for example the vascular cambium, storage parenchyma, cotyledons or mesophyll. In contrast, the explant such as stem, hypocotyl, whole embryo may be extremely heterogeneous and contain a wide range of cell types. A tissue such as a piece of stem or hypocotyl is typically a complex of differentiated cell types with differing sensitivities to imposed stimuli. The act of bringing such a heterogeneous fragment into contact with a complex medium, designed to promote callus formation, may simulate the wide variety of cells with differing proliferative capacity, ploidy level, and physiology within the population.

2.2.2 Sub culture

Wounding induces proliferation at the damaged surface of an intact plant which may result in the formation of a callus. Such a callus usually persists for only a short time and rapidly becomes infiltrated with polyphenolic substances, which seal off the wound from the environment. In order to sustain cell proliferation it is necessary to remove the developing callus and place it in culture in the presence of growth promoting substances.

Changes during proliferation and growth : Plant tissue cultures tend to lose the ability to differentiate with successive sub-cultures. Some cultures apparently lose the ability to differentiate and produce recognizable structures, but this can be restored by a change in the hormonal balance in the medium. Skoog and Miller (1957) has shown that an apparently unorganized tobacco callus can be induced to form either more callus or roots or shoots simply by effecting minute changes in the auxin : cytokinin concentration to which the tissue is exposed in the culture medium.

Chaturvedi and Mitra (1975) have demonstrated a shift in the morphogenetic pattern in callus tissue during prolonged culture. Stem callus only produced shoots in short term culture but began to produce embryoids after prolonged culture. It have been shown that embryogenic potential of carrot callus declines with time and this may be altered by changing the composition of the medium. The ability to differentiate into roots and shoots may also be lost with time in culture. Wilson and Street (1975) have shown that freshly initiated culture of *Hevea brasiliensis* will produce roots spontaneously but this property is lost during serial sub-culture.

Biochemical behavior during proliferation : Metabolic patterns in calluses may be modified during culture. The isoenzyme patterns of several proteins in *Phaseolus vulgaris* changed during a growth cycle. For examples glutamate dehydrogenase change from a pattern of five to a single electrophoretic band after sub-culture and then gradually returned to five towards the end of the culture period. (Arnison and Ball, 1974).

The failure of tissue culture to accumulate particular compounds implies not a loss of bio-synthetic potential but merely a failure to realize such potential under the conditions used. Key enzymes in a biosynthetic sequence that are observed in the initial isolate may be lost during serial sub-culture or at least are present in such small amounts as to reduce the flow of materials through the pathway below a detachable level. The effect of such lesions may be to lead to an accumulation of intermediates in unusual quantities, or to divert precursors to the synthesis of products not normally encountered , and these appear to be in the minority. In other cultures, the components of a particular biosynthetic pathway persist and the synthetic potential is preserved through many sub-cultures.

2.2.3. Field Transfer

In vitro regenerated plantlets often show marked physiological and morphological differences as they are grown under conditions which differ widely from those of seedlings. These include high relative humidity, low light intensity, heterotrophy due to high sucrose concentration, poor aeration and hormonal imbalances. This situation leads sometimes to poor vascular connection through

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out the plantlet which is essential for its survival in the field. Xylem vessels sometimes close before rooting and the lack of vascular connections cause poor survival on transfer to the field (Grout and Aston, 1977).

Rooting of *in vitro* shoot often affects plant survival. Two patterns of root formation has been recognized. One consists of direct development of root primordia from cells associated with or in close proximity to the vascular system. The other is an indirect process where root is formed from callus tissue without any vascuar connection to stem (Sahay and Verma, 2000)

The proper development of leaves is one of the most important factors for survival in the field. Wetzstein and Sommer, (1982) observed that *in vitro* plants showed reduced palisade parenchyma and increased mesophyll air space and inadequate stomatal closure in water stress. A few days after transfer to the field the leaves resembled those of seed raised plants. This may be one of the reasons for the requirements of acclimatization procedures to adopt tissue culture plants to low humidity in the field.

2.3 Plant regeneration

Plant regeneration *in vitro* occurs *via* two development pathways: Embryogenesis and organogenesis which differs in initial formation of either a bipolar or unipolar structure.

In **somatic embryogenesis** a new individual with a bipolar structure (*i.e.* a rudimentary plant with a root/shoot axis) arises from a single cell and shows no vascular connections with its maternal tissue (Haccius, 1978). In contrast, **shoot organogenesis** is characterized by the production of a unipolar bud primordium with its subsequent development into a leafy vegetative shoot. The developing shoot establishes conducting connection with the maternal tissue. The shoot then becomes rooted *via* root primordia formation and subsequent root organogenesis (Brown and Thorpe, 1986).

2.3.1 Organogenesis

Plant regeneration response has been shown to be under genetic control is several crops such as red clover (Keyes *et al.,* 1980), alfalfa (Hernandez-Fernandez

and Christie, 1989), Sunflower (Sarrafi *et al.,* 1996). The evidence indicates that morphogenetic competence *in vitro* is dependent on the culture environment and the physiological and organizational state of the cells (i.e. callus, cells and protoplast) (Cornejo-Martin *et al.,* 1979, Vasil, 1985). Different factors control morphogenesis in vitro and no theory clearly explains all the responses till date (Sahay and Verma, 2000).

Several studies have shown that success in *in vitro* organogenesis is largely dependent on following factors :

  (a)   Explant
  (b)   Genotype
  (c)   Medium composition

Manipulation of these factors leads to the initiation of organized development and ultimately, to dramatic structural changes.

(a) Explants : Plant consists of a heterogeneity of cell phenotypes which arise as a consequence of differentiation and development. This heterogeneity is evident from observation on tissue history, DNA (qualitative and quantitative) and studies on gene expression as manifested by changes in protein and isozyme production during differentiation and development (Nagl, 1978, 1979; Raghavan, 1983).

Changes in gene expression, and hence cell phenotype, may have major influence on a cell's capacity to produce a callus or regenerate plants *in vitro.*

Wide variety of plant parts have been used as the tissue source for cultures by various authors. The type of explant used to initiate *in vitro* cultures appears to be a critical factor in determining the capacity of cells and tissues for regeneration (Maddock, 1985; Vasil, 1987).

Several factors have been identified that are important for explant selection (Murashige, 1974; George and Sharrington, 1984). Some of the factors which affect callusing are as follows :

- the organ serving as explant and its physiological and ontogenetic age
- the season of explant excision

- the size of the explant
- Explant orientation on media
- pretreatment
- inoculation density *etc.*

In forages, fast growing meristematic tissues derived from embryos and seedlings are reported to be more responsive in cultures than those from the mature plants (Novak and Konecna, 1982; Lu *et al.*, 1982a,b; Ahuja *et al.*, 1983). In graminaceous species consistent shoot formation has been obtained mainly from callus induced from very immature material such as zygotic embryos and young inflorescences (Maddock, 1985; Vasil and Vasil, 1984; Vasil, 1985, 1987). Young inflorescences were more responsive than seeds in *Poa pratensis* for regeneration (Van der valk *et al.*, 1989).

In *Indigofera teysamni* it was found that high dose of BAP (upto 5 mg/L) induced more callus from leaf and shoot explants of seedlings. However, no such response was seen in mature leaf disc explant. The frequency of shoot production depends upon the physiological age of the explant, the older the tissue, the lower the frequency of shoot production (Ayyappan and Rajkumar, 1988). The importance of the age of explants in determining the morphogenetic expression of pea leaflet has also been emphasized (Morginski and Kartha, 1981).

(b)**Genotype** : The genotype is one of the major factors in determining the organogenic response. Species, cultivar and plant dependent regeneration has been reported in many species. A large number of legumes show genotype- specific regeneration as indicated in studies on *Medicago* (Bingham *et al.*., 1975; Phillips, 1983; Mitten *et al.*, 1984); *Trifolium pratense* (Keyes *et al.*, 1980; Bhojwani *et al.*, 1984; McLean and Nowak, 1989); *Trifolium* sp. (Webb *et al.*, 1984); *Pisum* (Malmberg, 1979, Kunakh *et al.*, 1984); *Cajanus* (Kumar *et al.*, 1983, 1984 a, b); *Vitis (Clog et al., 1990); Allium* (Rauber and Grunewaldt, 1988). Individual genotype of the same alfalfa variety have been shown to require different phytohormone and salt concentrations for regeneration (Kao and Michayluk, 1981).

(C)Culture medium composition : A successful organogenesis *in vitro* involves medium optimization as one of the first steps as there is no single medium that must be used for a given species or type of culture. The chemical composition and physical make up of the nutrient medium are determining factors in plant regeneration.

The ratio of auxin and cytokinin has been reported by various authors to play a significant role in organogenesis. Classical work of Skoog and Miller (1957) suggested that quantitative interactions between growth regulators, especially auxin and cytokinin and other metabolites provide a common mechanism for the regulation of all types of growth including organ formation. Manipulation with stem pith-derived tobacco callus showed that a high ratio of auxin to cytokinins in the nutrient medium favoured root formation, the reverse favored shoot formation, and an intermediate ratio promoted callus proliferation. However, this approach can not be demonstrated in all species. To induce plant regeneration, calli are generally transferred to media with lower auxin levels, which may be further improved by the addition of cytokinins. Further, studies confirm the role of endogenous auxin-cytokinin ratio in organ control. Transposon insertions which inactivate T-DNA genes 1 and 2 involved in auxin biosynthesis decrease the auxin/cytokinin ratio in the tissue and shoot forming teratomas are formed as a consequence (Garfinkel *et al.,* 1981; Inze *et al.,* 1984). No callus was induced in *Vitis* when BA was absent which indicate that BA is required for shoot initiation (Clog *et al.,* 1990). Inactivation of a single T-DNA gene involved in cytokinin biosynthesis increases the auxin / cytokinin ratio and root farming teratomas are produced (Garfinkel *et al.,* 1981; Akiyoshi *et al.,* 1984). In *Cajnus cajan* the relative effectiveness of different cytokinins for multiple shoot formation was found to be in order - BAP - Kinetin -.Zeatin - Adenin (Shiva Prakash *et al.,* 1994), while BAP was found to be better than 2-ip for callus induction in *Indigofera* (Ayyappan and Kumar, 1989). IAA and IBA were ineffective at all concentrations in inducing roots, whereas NAA showed better response in *Bixa* sp. (Sharon and D'Souza, 2000)

No influence of the amount of NAA and IAA on explant response was seen in *Allium* species (Rauber and Grunewaldt, 1988). In two legumes, *Arachis* and

Cajanus frequency of shoot regeneration was dependent on the type of auxin present in the medium (Eapen and George, 1993).

2.3.2. Somatic embryogenesis

Somatic embryogenesis can be defined as the process in which a bipolar structure arises through a series of stages characteristic for zygotic embryo development and having no vascular connection with the parental tissue (Ammirato, 1987; Terzi and Loechiavo, 1990; Raemakers *et al.,* 1995). The development of somatic embryos closely resembles that of zygotic embryos both morphologically and temporally. However, Somatic embryos, in contrast to zygotic embryos, grow and differentiate continuously, apparently activating the shoot and root apical meristem with no obvious quiescent state (Zimmerman, 1993).

The similarity between zygotic and somatic embryogenesis is both striking and remarkable. The fact that structurally and developmentally normal embryos can develop from somatic cells indicate that the genetic programme for embryogenesis are totally contained within the cell and can function completely in the absence of gene products from the maternal environment (Zimmerman, 1993).

Somatic embryos have a bipolar structure in which shoot and root meristems are directly connected with no interruption by non differentiated callus tissue (Lorz *et al.,* 1988). Although plants regenerated through tissue culture in some species are less variable than their original donor or explant sources (Feher *et al.,*1989; Gmitter *et al.,* 1991), enhanced variability (both phenotypic and cytological) from embryogenically regenerated plants over organogenetically regenerated plants from the same explant source have been documented (Armstrong and Phillips, 1988; Browers and Orton, 1982; Ahloowalia and Maretzki, 1983; Karp and Maddock, 1984). The variability may be caused by a constant mutation rate per cell generation with a multiplicative effect due to an increased number of generation *in vitro* (Peschke and Phillips, 1992).

In somatic embryogenesis, the embryoid is often derived from a single cell although evidence for a multicellular origin has also been obtained. Single cell

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origin has been observed for pearl millet (Vasil and Vasil, 1982). Sugarcane (Ho and Vasil, 1983). Multicellular origin has been reported in maize (Vasil *et al.*, 1985), Celery (Browers and Orton, 1982). Somatic embryos have been an excellent source for secondary embryos. It is associated with less of integrated group control of cells organised in the somatic embryos. Some cells break away from group control and initiate new somatic embryos (Williams and Maheswaran, 1986).

The first observations of *in vitro* somatic embryogenesis were made in *Daucus carota* (Reinert, 1958; Steward *et al.*, 1958). Since then somatic embryogenesis has been described in more than 200 species (Evans *et al.*, 1981; Tulecke, 1987; Raemakers *et al.*, 1995). Virtually every plant organ has been shown to form embryos.

Embryos of the first recognizable stage, globular stage, generally grow out of cell cluster within 5 to 7 days after transfer to auxin free medium in carrot. After 2 to 3 more days of isodiametric growth, the globular stage is followed by an oblong stage (Schiavone and Cooke, 1987), which signals the shift from isodiametric to bilaterally symmetrical growth and the beginning of the heart stage. This transition is clearly marked by the out growth of two cotyledons, the elongation of the hypocotyl and the beginning of the radicle. By three weeks after induction, plantlets can be identified (Zimmerman, 1993).

In most cases somatic embryos develop up to pre-embryonic masses (PEM's) or globular embryos, without differentiation into organs, before they are subjected to secondary embryogenesis (indirect embryogenesis). In other cases embryos develop up to maturity (direct embryogenesis) (Raemakers *et al.*, 1995).

The nutritional requirements for somatic embryogenesis are not well understood. They are neither specific nor exclusive, since various recipes produce similar results. However, auxin and reduced nitrogen are believed to be the prime controlling factors in somatic embryogenesis. (Laxmi *et al.*, 1999).

Evans *et al.*, (1981) and Sharp *et al.* (1980, 1982) proposed the concept that somatic embryogenesis is initiated by either of two cell types "pre embryogenic determined cells"(PEDCs) and "Induced embryogenic determined cells" (IEDCs).

PEDCs are already determined for the embryogenic pathway and await only the synthesis of an inducer (or removal of an inhibitor) to resume independent mitotic divisions and express their embryogenic potential. PEDCs are found in embryonic tissues, in the nucellus and embryo within the ovules. IEDCs require redetermination to the embryogenic state, generally by exposure to specific growth regulators. IEDCs occurs in anthers cultures and in callus cultures particularly after treatment with auxins. Once the embryogenic state has been induced there appears to be no fundamental difference between IEDCs and PEDCs.

The potential application of somatic embryogenesis in plant improvement depend to a large extent on whether proliferation is from PEDCs or IEDCs. Direct somatic embryogenesis from PEDCs without cellular destabilization and redifferentiation appear to produce relatively uniform clonal material. Indirect pathway through IEDCs where an intervening destabilized callus phase is present tends to generate a higher frequency of somaclonal variants (Williams, 1987).

Hormonal role in somatic embryo development : Many authors (Schiavone and Cooke, 1987; Michalczuk *et al.* 1992; Zimmerman, 1993) have shown that auxin appears to play important roles both in the induction of embryo development in culture and in the subsequent elaboration of proper morphogenesis in embryo development.

In vitro development of somatic embryos in carrot was reported to be a two step process, each requiring a different medium. The callus is initiated and multiplied in an auxin rich medium called 'Proliferation medium', in which callus differentiates localized groups of meristematic cells called "embryogenic clumps"(EC). In repeated subcultures on the proliferation medium the ECs continue to multiply. However, if the ECs are transferred to a medium with very low auxin or no auxin, they develop into mature embryos. Thus, proliferation medium could be regarded as the induction medium for somatic embryogenesis (Snug and Okimoto, 1981) and each EC a disorganised embryo (Bhojwani and Razdan, 1983).

The promotive effects of NAA (Lazzeri *et al.,* 1987); 2,4-D (Hazra *et al.,* 1989) and picloram (Kysely and Jacobson, 1990) on *in vitro* induction of somatic

embryos in cultures of legumes have been demonstrated (George and Eapen, 1994). In carrot, Halperin (1970) reported initiation of embryogenesis by BAP and Gibberllins. However, Fujimura and Komamine (1975) recorded promotive effect of cytokinins on embryogenesis.

The role of exogenous auxin in somatic embryo induction depends on the nature of explant (Ammirato, 1983). The exposure duration of exogenous auxin vary with explant source and species (Dudits *et al.*, 1993). Raemakers *et al.*, (1995) while reviewing primary and secondary embryogenesis opined that in general, auxin and or auxin/cytokinin supplemented media are used in somatic embryogenesis of Gymnosperms and monocot Angiosperm species. In certain, dicot species also growth regulator free and/or cytokinin supplemented media can initiate embryogenesis.

Gene expression during Somatic embryogenesis : The dramatic transition from unorganized callus cell growth to somatic embryo development suggest that a substantial reprogramming of gene expression, presumably occurring at the transcriptional level, dictates the development switch (Fujimura and Komamine, 1980; Zimmerman, 1993).

Several genes that are preferentially expressed in somatic embryos belong to a class of hydrophillic proteins called Late Embryogenesis Abundant (LEA) proteins (Dure *et al.*, 1989; Galiba *et al.*, 1986). Most of the LEA transcripts increase significantly in somatic embryos at the heart stage (Kiyosue *et al.*, 1992, 1993; Wurtele *et al.*, 1993).

It was reported that some extracellular proteins (EP) are secreted that could further induce somatic embryogenesis (De Jong *et al.*, 1992; Smith and Sung, 1985). It has been suggested that these secreted proteins play a role in the regulation of cell expansion / which is critical to the maintenance of the integrity of epidermal layer in embryos and to the proper establishment of shape and form (Van Engelen and De vries, 1992; Sterk and De vries, 1993).

2.4 Somaclonal variation :

Assembly of genetic variability is vital for improvement of crop plants. Somaclonal variation can be utilized for the improvement of specific traits, particularly where they are lacking in available germplasm. Plant cell culture has provided a new and exciting option for obtaining increased genetic variability relatively rapidly.

Somaclonal variation was defined by Larkin and Scowcroft (1981) as the genetic variation displayed in tissue culture regenerated plants and their progeny. Peschke and Phillips (1992) while reviewing the subject included in it any genetic, cytogenetic or molecular changes produced during tissue culture or plant regeneration. The somaclonal variation, alongwith the corresponding changes observed in tissue culture *per se* has been documented by numerous authors in a large number of species (Karp, 1995; Peschke and Phillips, 1992; Evans and Sharp, 1986). Tissue culture regenerated variants have also been called calliclones (Skirvin and Janick, 1976), pheno variants (Sibi, 1976), protoclones (Shephard *et al.*, 1980) and subclones (Cassells *et al.*, 1991). The utilization of new genetic variability induced either spontaneously or artificially during the culture process has become one of the major objectives of tissue culture.

The widespread occurrence of somaclonal variation in wide variety of plant species has been extensively documented. Somaclonal variation does not appear to be species or organ specific and many of the plant traits for which genetic variability is generated during tissue culture cycle are of agronomic value and can thus provide a valuable adjunct to plant improvement.

Scowcroft (1984) emphasized that plant tissue culture *per se* appears to be an unexpectedly rich and novel source of genetic variation. The first substantive example of somaclonal variation was recorded in sugarcane in Hawaii (Heinz and Mee, 1969). Variation was observed in morphological, cytogenetic and isozyme traits (Heinz, 1973). Subsequently somaclones were identified with increased resistance to various diseases such as Fiji disease virus, downy mildew and eye spot disease (Krishnamurthi, 1974; Krishnamurthi and Tlaskal, 1974; Heinz *et al.*, 1977), for high sucrose concentration (Heinz and Mee, 1971; Heinz *et al,* 1977). In

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potato, variants have been reported for growth habit, tuber colour, maturity date, tuber uniformity and disease resistance (Shephard *et al.*, 1980). In some cases the somaclonal variants have been field tested and found unstable (Larkin and Scowcroft, 1983), while in other cases stable variants have been selected (Krishnamurthi, 1982) including lines resistant to Fiji disease and downy mildew.

In large number of cases the variants were not useful mainly because of following reasons - negative variation, not novel variation, unstable variants which change after selfing or crossing. This is a novel means of making beneficial selections. However, while serving as a resource seeking desirable traits, it often serves as a nuisance to those attempting to create defined changes in plants through transformation or who wish to preserve germplasm *in vitro*. The tool has been most successful in crops with narrow genetic bases, where it can provide a rapid source of variability for improvement (Karp, 1995).

The study of the heritability of somaclonal variation has revealed that it can result from either genetic, epigenetic on nongenetic change (Meins, 1983). Non genetic variation includes chimeral breakdown, physiological effects, and the elimination of virus or virus like agents (Cassells, 1985).

Genetic variants are heritable variants as judged from studies on Mendelian inheritance of variant traits (Orton, 1984; D'Amato 1985). Such variants can be exploited for further studies.

Epigenetic change results from a change in gene expression and not gene mutation (Chaleff, 1981; Meins, 1983). Such variants, therefore, revert when sexually propagated. Epigenetic variation can be transient or temporary in later generations even when the material is asexually propagated. This variation includes phenotypic changes that involve expression of specific genes (Hartman and Kasler, 1983). Because explants adopt to an *in vitro* environment in stepwise fashion by becoming more juvenile, the resulting calli may vary in maturity from juvenile, to fully mature. Plants regenerated from these tissues also vary depending on the developmental stage progression of the tissue when the stimulus to regenerate is applied (Skirvin *et al.*. 1994). Shoot regeneration from dedifferentiated callus can produce an immature, unstable clone that may

eventually revert to the original parental clone. Examples of epigenetic variation include partial fertility, male sterility, or transient dwarfism (Mc Pheeters and Skirvin, 1989; Moore *et al.* 1991) that are associated with a carryover of growth regulators from the tissue culture medium, tissue or cellular habitation involving the loss of auxin, cytokinin or vitamin requirements by callus (Skirvin, 1978; Meins, 1989; Jackson and Lyndon, 1990).

Somaclonal variation results from both preexisting genetic variation within the explants and variation induced during the tissue culture phase (Evans *et al.,* 1984). Preexisting variation can evolve from non-uniformity in multicellular explants (multiple types of cells such as phloem, parenchyma, cortex and xylem parenchyma).

Factors affecting occurrence and frequency of somaclonal variation :

It can not be said that *in vitro* culture will always give rise to variation. In fact, a number of factors are identified that influence occurrence and frequency of variations. These include : Explant source, Explant genotype, Culture age, Culture conditions, Ploidy level and Karyological aberrations

(1) Explant source : The source of explant has been considered most often as a critical variable for somaclonal variation (Skirvin *et al.,* 1994). Since all explants are not equal in term of regenerability, it is likely that different selective pressures would be exerted against different explants. This could result in different frequencies and spectrums of somaclonal variation among plants from different explants. Thus, differences in both the frequency and nature of somaclonal variation have been reported when regeneration is achieved from different tissue sources. However, while reviewing the topic Peschke and Phillips (1992), preferred to make two generalizations.

(1). Meristems cultured without a state of differentiation will produce little or no variation compared to when a dedifferentiated state is induced (Karp and Bright, 1985; Bayliss, 1980; D'Amato 1985; Potter and Jones, 1991). Highly differentiated tissues (roots, leaves and stems) produce more variation than explants with preexisting meristems (axillary buds and shoot tips) (Duncan, 1997).

(2) Differences in the stability of tissue cultures produced from different explants can often be traced to variability preexisting in the explant. The most recognized case is polysomaty (wherein diploid and polyploid cells coexist in the same tissue), that may be found in over 90% of plant species (D'Amato, 1985, 1989). Van der Bulk *et al.* (1990) reported a high degree of polyploidy (58%) in plants regenerated from cotyledon, leaf in Tomato. They also demonstrated that the hypocotyl is polysomatic whereas the other explants tested (cotyledon and leaf) showed diploid cells. Similarly, Sree Ramulu *et al.* (1986) found that higher protoplast-derived potato plants showed a higher frequency of chromosomal and phenotypic alteration when the protoplasts were obtained from a chromosomally variable cell suspension than when they were freshly isolated from diploid tissue. In tobacco, 0.1-1.87% of the variation was present originally in the mesophyll protoplast, whereas 1.4-6.0% of the variation was attributed to tissue culture stress.

(2) Genotype of Explant : The genotype of plants used for somaclonal variation is an important variable which can influence both the frequency of regeneration and somaclones (Evans *et al.,* 1981; Evans and Sharp, 1986). Frequency of polyploid regenerants in 18 varieties of rice were compared and multiploids were recovered from the indica varieties but not in the Japonica varieties. Similarly, the frequency of chlorophyll deficiency mutants varied significantly between the two types of rice varieties (Sun *et al.,* 1983) Varietal (cultivar) differences have also been reported in oats (Mc Coy *et al.,* 1982), maize (Zehr *et al.,* 1987), wheat (Mahmand *et al.,* 1990) and *Medicago* sp. (Nagarajan and Walton, 1987). The reasons for such genotypic differences can be attributed to preexisting variation (Morrish *et al.,* 1990) (such as polysomaty etc). There might be differences in the degree to which the tissue culture environment disrupts the cellular environment of a particular line (Peschke and Phillips, 1992).

Some cultivars may have genes that control tissue culture regeneration; others may not have the genes for regeneration or the genes controlling phytohormone signals, but the trait can be transferred *via* traditional breeding methods into these genotype (Smith and Quesenberry, 1995). Genes on different chromosomes are involved in control of callus growth (Shimada and Makino, 1975; Baroncelli *et al.,* 1978) and in the regeneration of shoots (Galiba *et al.,* 1986;

Mathis and Fukui, 1986; Mathis *et al.*, 1988; Kaleikau *et al.*, 1989), suggesting a polygenic system (Henry *et al.*, 1994) governing induction of cells involved in embryogenesis. Certain genes have a major effect on somatic embryogenesis and regeneration (Brown and Atanassov, 1985), whereas the lack of certain genes on other chromosomes may suppress embryogenesis (Henry *et al.*, 1994). Genetic control of somatic embryogenesis in alfalfa (*Medicago sativa* L.) was found to be under the control of two dominant loci (Crea *et al.*, 1995).

Embryogenic callus production, shoot regeneration and root regeneration are controlled by recessive genes and are inhibited by dominant suppressers. Production of non-embryogenic callus is determined by dominant genes that can give an additive effect. The lack of *in vitro* response is caused by at least two interacting genes acting in a suppressive fashion in rye.

Genetic analysis of regeneration ability has also revealed that the trait can be controlled by a few genes (one to three loci) with quantitative and highly heritable effects (Reish and Bingham, 1980; Charmet and Bernard, 1984; Brown and Atanassov, 1985; Mclean and Nowak, 1989; Nadolska-orcayk and Malepszy, 1989), polygenic control strongly influenced by environment (Tomes and Smith, 1985), polygenic control with little environmental influence (Rakoczy-Trojanowska and Malepszy, 1995), dominant genes (Komatsudo *et al.*, 1989; Nadolska- Orczyk and Malepszy, 1989; Reish and Bingham 1980) having positive heterotic effects (Flehinghaus *et al.*, 1991).

(3) **Culture age :** Increase in somaclonal variations of all types have been reported with an increase in culture age (McCoy *et al.*, 1982; Benzion and Phillips, 1988; Muller *et al.*, 1990). The effect of age has been attributed to one or a combination of following causes by Peschke and Phillips (1992).

- Culture *per se* becomes more prone to change as it gets older, *i.e.* , the mutation rate per cell generation increases.
- A number of mutations takes place early but detected only when a sufficient number of mutant cells have accumulated.
- Mutations occurring at an early culture age are actively selected.
- Increased polyploidy (Murashige and Nakano 1967; Colijn- Hooyamns *et al.*,

1994).

Benzion and Phillips (1988) analyzed pedigrees of a large number of callus lines and found that cytological aberrations detected in plants regenerated after many months in culture could often be traced back to a common subculture point early in the culture process. In contrast, Fukui (1983) reported that mutations detected in rice regenerants appeared to have occurred at various stages throughout the culture process.

Barbier and Dulieu (1983) using a genetically marked explant source, have shown that while most genetic changes occur in the first few mitoses in culture, some genetic changes increase with the duration of culture. Lorz and Scowcroft (1983) showed that by doubling the duration of culture, the frequency of genetic changes increased from 1.4 to 6% in protoplast of heterozygous clones.

It has been increasingly indicated by several authors that extended culture produces an elevated frequency of somaclones. Somatic hybrids are found to be a richer source of variability than sexual hybrids (Evans *et al.,* 1982).

It has been reported that as callus tissue increases, the morphogenesis potential decreases, whereas, the frequency of albino shoots and only root producing callus increases (Wen *et al.,*1991) The highest frequency of chlorophyll variations found in regenerated plants from inflorescence cultures come from 210+ days old cultures (Cai *et al.,* 1990).

The degree and extent of polyploidy in an *in vitro* culture tends to increase progressively with increasing age of the primary explant or callus or under particular hormonal regimes (Melchers and Bergman, 1958; Mitra and Steward, 1961; Blakely and Steward, 1964; Murashige and Nakano, 1967; Demoise and Partanen, 1969; Mehra and Mehra, 1974). An important mechanism of polyploidization *in vitro* appears to be restitution nucleus formation due to spindle failure and chromosome lagging at anaphase (Bayliss, 1973).

Among the various ploidy levels occurring in cultures of somatic plant tissues of special interest are odd-ploid chromosome numbers; haploid, triploid, pentaploid *etc.* Haploid and tripoid mitoses are reported to occur in *in vitro*

cultures of *Happlopappus gracilis* (Mitra and Steward, 1961; Shamina, 1966); a callus strain that showed 13% triploid mitoses (3x=6) at the fourth month of culture (Shamina, 1966), when examined several years later, was found to comprise a wide range of ploidy from diploidy (Sidorenka and Kunakh, 1970).

(4) **Culture conditions :** Growth regulator composition of the culture medium has been indicated to influence frequency of karyotypic alteration in cultured cells (Bayliss, 1975; Deambrogio and Dale, 1980).

Hormonal factor : Callus is initiated *in vitro* on cut or exposed cell surface in contact with a growth medium. Callus proliferation is a wound response. Excision of the explant stimulates the wound response *in vivo* which can be enhanced by growth regulators (McClintock, 1984). Most plant growth regulators, and specially, 2, 4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine have been implicated in tissue culture induced variability (D'Amato, 1985; Evans, 1988; Grierback *et al.,* 1988; Shoemaker *et al.,* 1991; Skirvin *et al.,* 1994).

The primary event causing tissue culture-induced variability may be cell cycle disturbance (Peschke and Phillips, 1992) caused by exogenous hormone effects (Bayliss, 1977a, b; Bhaskaran and Smith, 1990; Liscum and Hangarter, 1991) or nucleotide pool imbalances (Jacky *et al.,*1983). Auxins can produce rapid disorganised growth during callus induction that may lead to genetic instability through asynchronous cell division (Gould, 1984; Lee and Phillips, 1988). Increased thymidine can enhance chromosome breakage (Ronchi *et al.,* 1986; McClintock, 1978), chromosome breakage could lead to aneuploidy (*via* chromosome fragment loss), activation of transposable elements, methylation changes (Grafstrom *et al.,* 1984), initiation of SOS response resulting in single base changes (Walker, 1984), and an error-prone repair system (Burr and Burr, 1988). Exposure of plant cells to culture may provide conditions for disruption of genomic stability (due to the behaviour of repeated DNA sequences) leading to release of variability (Bhaskaran, 1987).

Kinetin (K) acts as a trigger for mitosis in endoreduplicated cells (Torrey, 1961; Van'T HOF and Macmilan 1969) and in cultured pea root cortex cells it can induce endoreduplication prior to mitosis (Libbenga and Torrey, 1973). Selective

induction and maintenance of mitosis in cells of different ploidy levels was first demonstrated in pea root segments, cultured *in vitro* on Shigemura's synthetic medium, the diploid cells only proliferated; when K (or yeast extract) plus 2, 4-D were added to the medium, the tetraploid cells were selectively stimulated to mitosis and the proliferating fraction of the culture consisted of tetraploid cells only (Torrey, 1961, 1967; Matthyse and Torrey, 1967). Direct as well as inverse correlation have been reported between polyploidy in tissue cultures and the presence of 2, 4-D in the medium. Some authors regard it as a direct inducer of polyploidy (Sunderland, 1977; Mitra and Steward, 1961), others considered it as a factor selectively favouring the divisions of polyploid cells. An inverse relationship between the concentration of 2, 4-D and the degree of polyploidization was reported in *Pisum sativum* (Kallak and Yarvekylg, 1971) and in *Haplopappus gracilis* and *Vicia* sp. (Singh and Harvey, 1975). Since a combination of auxin and cytokinin is essential for DNA synthesis and mitosis (Skoog and Miller, 1957) their quantitative ratios in a culture medium can greatly influence the composition of the proliferating cell population, as shown with *H. gracilis* callus. Bennici *et al.* (1971) noted that increasing concentration of NAA and kinetin decreased the frequency of polyploid mitosis.

(5) **Ploidy factor :** Regenerated plant variability is higher among polyploid and high - chromosome number explant sources (Heinz and Mee, 1969, 1971; Creissen and Karp, 1985) than among lower ploidy and low-chromosome number species. Spontaneous doubling is a common type of chromosome aberration in many diploid dicotyledonous regenerated plants (Murashige and Nakano, 1966, 1967) whereas tetraploids regenerated from callus or protoplasts often have chromosome structural changes, aneuploidy, and chromosome doubling (Bingham and McCoy, 1986). Polyploidy in tissue culture is generally the product of either endopolyploidization or nuclear fusion (Sunderland, 1977; Bayliss, 1980). Aneuploid reinsertions are more common in polyploid than in diploid of same species (Peschke and Phillips, 1992).

(6) **Karyological Aberrations:** Chromosome abnormalities of *in vitro* regenerated plants can include numerous karyological changes (Karp and Bright, 1985; Lorz *et al.*, 1988). Chromosome breakage and its consequences (deletions,

duplications, inversions and translocations) cause common aberrational events (Sacristan, 1971). The breakage positions are not random, but involve late replicating chromosome regions characterized by heterochromatin (Lima-de-Faria, 1969; Sacristan, 1971; McCoy et al.. 1982; Lapitan et al.., 1984; Marata and Orton, 1984; Johnson et al., 1987; Lee and Phillips, 1987; Benzoin and Phillips, 1988). The role of heterochromatin in causing chromosome breakage may be due to later than normal chromosome replication in tissue culture, with the chromatids being held together and creating a stress that results in breakage between the centromere and late-replication region (McCoy et al., 1982; Lee and Phillips, 1988). Tissue culture has also resulted in increased frequencies of sister chromatid exchange (Dolezel and Novak, 1986; Dimitrov, 1987; Dolezel et al., 1987) and somatic crossing over involving two homologues instead of two chromatids (Lorz and Scowcroft, 1983). Somaclonal variation may not be random because specific loci may have higher mutation rates than others during the in vitro process (Xie et al., 1995).

Positive attributes of somaclonal variation :

- It is a cheaper technique as compared to somatic hybridization and transformation, DNA recombination.
- It results in a rich source of genetic variability.
- It is not necessary to have identified the genetic basis of the trait, as in the case of transformation where isolation and cloning is required.
- Novel variants have been reported among somaclones, and genetic (Comptan and Veilleux, 1991) and cytogenetic evidence indicate that both the frequency and distribution of genetic recombination event can be altered by passage through tissue culture.
- Plant with genetic variability can be directly transferred to the field and evaluated as part of an ongoing programme.
- Somaclonal variation also hold promise as an adjunct to protoplast fusion (Evans and Sharp, 1986).

This suggests that variation may be generated from different areas of the genome than those that are accessible to conventional and mutation breeding. It is

not a precision tool and only minimal control can be exercised over its operation, and can be an important tool in crops with narrow genetic bases.

2.5. Chromosomal aberrations in cultured cells and regenerated plants

Variation in chromosome number and structure has been observed among cultured cells and regenerated plants as indicated by several workers (Bayliss, 1980; D'Amato, 1985; Lee and Phillips, 1988; Peschke and Phillips 1992). These variations can be grouped in following types :

1. **Polyploidy/Aneuploidy -** Variant plants with altered chromosome number have been reported by several authors. Polyploidy is the most frequently observed chromosomal abnormality and is generally explained as the product of either endopolyploidization or nuclear fusion (Sunderland, 1977; Bayliss, 1980). Aneuploidy may be caused by nondisjuction, lagging chromosomes, aberrant spindles and chromosome breakage producing dicentric and acentric chromosomes (Sunderland, 1977), or polyploidy followed by chromosome elimination (Balzan, 1978).

Aneuploidy is better tolerated in polyploid species than diploid ones, due to the greater imbalance of genetic material in the diploid situation. Changes in chromosome number are commonly associated with reduced fertility and with altered genetic ratios in progeny of self fertilized plants (Evans and Sharp 1986). Presence of polyploidy (up to 64 chromsomes instead of normal 2n=4), aneuploidy and anaphase abnormalities as well as morphologically altered chromosomes in cells of predominantly normal diploid *Haplopappus* culture has been reported (Mitra and Steward, 1961).

In culture, irregular polyploidy or aneuploidy may occur in different parts of the culture, in localized areas. This reflects that replication or multiplication of chromosomes may have preceded in different cells at different rates (Krikorian *et al.* 1983). In some cell lines of daylily plants, the majority of cells in each population is represented by a zone of connecting chromosome numbers or gradation in numbers (Krikorian *et al.,* 1983).

D'Amato (1972) and Vant'of (1974) supported the view that proliferating —
or potentially proliferative cells in a species are genetically determined to arrest in
a certain cycle phase; for example 'G' phase. It seems plausible that, in a given
species, the cell cycle control which operates *in vivo* also operates *in vitro*. Leaf
callus of *Crepis capillaris*, when analyzed cytologically and cytophotometically,
was found to consist of diploid cells only up to one year of culture (Reinert and
Kuster, 1966); but in the course of time, polyploidy began to appear and increased
with time to reach 28% after 20 months (Sacristan, 1971).

In the majority of plant species, differentiated tissues *in vivo*, contain
endopolyploid nuclei, that is nuclei whose chromosomes at interphase have
undergone one to several duplications : up to twelve in the polytene chromosome
cells of the suspensor of *Phaseolus caccineus* and *P. vulgaris* (Nagl, 1974).
Polyploid and aneuploid regenerated plants or shoots have occurred in cell or
tissue cultures of many species (D'Amato, 1977). Plants with doubled
chromosome number were produced from over 100 sugarcane clones (Heinz *et al.*,
1977). Nine octoploids were found among 200 regenerated alfalfa plants from
tissue cultures initiated from tetraploid alfalfa (*Medicago sativa)* by Saunders and
Bingham (1972). Genotypic effect was attributed to the observation in which
regenerants of one variety of tobacco were diploid, where are those of another
were tetraploid (Dulieu, 1972).

2. **Chromosomal breakage :** McCoy *et al.,* (1982) proposed the hypothesis for
role of heterochromatin in causing chromosome breakage due to its late
replication. In addition, this could also result in nondisjunction and subsequent
aneuploidy. Sacristan (1971) found that in *Crepis capillaris* callus culture, the
SAT-chromosome was involved in 82% of the rearrangements, with the break
point (s) corresponding to a region of late DNA synthesis. Chromosome breakage
in regions of late DNA synthesis (likely heterochromatic) seems consistent with
observations of tissue culture induced chromosome rearrangement in many cases
(Armstrong *et al.,* 1983; Ashmore and Gould, 1981; Lee and Phillips, 1987, 1988).
In tissue culture of *Haplopappus gracilis* frequent occurrence of acentric
fragments, microchromosomes, deleted chromosomes, dicentric chromosomes and
ring chromosomes have been noted (Singh 1975).

In cultures of somatic cells, simultaneous breakage of homologous chromosomes could lead to duplications and deficiencies whereas simultaneous breakage in nonhomologous chromosomes could lead to reciprocal interchanges (Lee and Phillips, 1987).

3. Nucleotide pool imbalance : The effect of nucleotide pool imbalance have been documented for a wide variety of species. Plant cells in tissue culture may be especially susceptible to dNTP (deoxyribonucleotide) pool imbalance because they can be serially transferred from depleted to fresh media almost indefinitely (Lee and Phillips, 1988).

4. Mitotic recombination : Various forms of mitotic recombination, including somatic crossing over and sister chromatid exchange (SCE) could produce several types of chromosomal rearrangements observed in tissue culture, especially if the exchange were asymmetric or between nonhomologous chromosomes (Larkin and Scowcroft, 1981). In *Haplopappus* culture, chromosomal arrangements strongly resembling chiasmata was reported in somatic cells during mitosis which, may represent mitotic crossing over or segregation (Mitra and Steward, 1961). Dolezel *et al.*, (1987) also reported that SCE frequency increase with a low concentration of 2, 4-D. Dimitrov (1987) observed that most of SCE is *Crepis capillaris* occurred at the junction between early and late replicating regions. Higher frequency of SCE was reported in callus in comparison to root tips (Dolezel and Novak, 1986).

5. Translocations : Translocations have been reported in cultured cell population of *Crepis* sp. (Sacristan, 1971), Carrot (Bayliss, 1975), *Allium sativum* (Novak, 1974, 1981).

2.6. Isozymic studies in somaclonal variants

In the broad sense, isozyme refers to any two distinguishable proteins that catalyze the same biochemical reaction. Isozymes are multiple molecular forms of an enzyme with similar or identical substrate specificities occurring within the same organism (Markert and Moller, 1959). This phenomenon came under extensive study after development of zymogram technique by Hunter and Markert

(1957). This technique involves electrophoretic separation of tissue extract, followed by demonstration of zones of enzyme activity using specific histochemical staining procedure applied directly to the electrophoretic medium. This techniques allows for the resolution of isozymes mainly on the basis of charge and size differences. It does not discriminate mutants that may have similar electrophoretic mobilities, but may differ in physicochemical properties.

Isozymes are direct gene products and are, therefore, less susceptible to modification by environmental factors. Since enzymes are coded for by genes, any disruption in the coding sequence even at the single base level could force variations in the expression of the enzyme, leading to an altered individual. Variation detected in isozyme studies have genetical basis, thus they are used as markers in many biological studies. The regeneration of whole plants from cultured cells has been an area of intense investigation. Isozymes may provide a unique tool in such studies.

Isozyme patterns have been used to detect changes during regeneration as they play a vital role in development and differentiation (Orton, 1983, Chawla, 1988). Isozyme study has been suggested as a valuable tool in identifying genetic and epigenetic changes (Michel, 1975). Isozymes constitute ideal markers for tissue and somatic cell genetic studies due to :

- The ease of detection
- the abundance of naturally occurring variants in most plant populations.
- Applicability to small amounts of tissue and crude extracts.
- the marker is mostly expressed in the "undifferentiated" state of a cell culture.
- Many loci express at all stages of the development.
- Genetic inheritance can be easily demonstrated. Most loci have Mendelian inheritance.

Isozymes are used primarily in plant tissue culture for physiological studies. Peroxidases in *Nicotiana* (Lee. 1971; 1972) and in *Pelargonium* culture (Lavee and Galston, 1968) were reported to vary in cultures. Definite qualitative changes in the isozyme patterns of peroxidase, esterase and acid phosphatase were observed in response to varying light and temperature conditions (McCown *et al.,*

1970).

Various authors have reported, isozymic variation during tissue culture. Variation in esterase and acid phosphatase was reported in tobacco callus culture (Bassiri and Carlson, 1979), in wheat and barley calli during differentiation (Chawla, 1988). Novel acid phosphatase bands were observed during cytodifferentiation in callus cultures of *Vigna* (De and Roy,1984). Isozyme studies as markers to distinguish between embryogenic and non-embryogenic calli in *Panicum maximum* was suggested by Alarmelu *et al.*, (1999). They found acid phosphatase to be organ and tissue specific. Isozyme pattern of esterases were found to vary with developmental stages. Twelve isoesterases were detected in the embryogenic calli, out of which only two are retained at the time of germination of embryoids into plantlets.

Study of different isozymes such as acid phosphatase in *Panicum maximum* (Alarmelu *et al.*, 1999), in other grasses (Lorenc-kubis and Marawiecka, 1985); esterases in guinea grass (Alarmelu *et al.*, 1999), in maize (Everett *et al.*, 1985) has been suggested for differentiating between embryogenic and non-embryogenic calli.

Variation in glutamate dehydrogenase was reported at various stages of callus growth in Phaseolus vulgaris (Arnison and Boll, 1974). They reported change from a pattern of five to a single electrophoretic band after subculture and gradual return to five at end of the culture period.

In hexaploid wheat, study of 17 somaclonal variants show chromosomal changes as well as change in ADH-1 phenotype (Davies *et al.*, 1986). Alteration in phosphoglucomutase and shikimate dehydrogenase phenotype were observed in regenerated celery plants, which were also correlated with karyotypic changes (Orton, 1983).

No variation was detected in protoplast derived Russet Burbank potatoes in a screening involving 13 enzyme systems (Sanford *et al.*, 1984). Similarly 180 regenerated garden pea plants showed same zymograns for esterase, glutemate dehydrogenase, 6-phosphogluconate dehydragenase and leucine amino peptidase

(Rubluo *et al.*, 1984). Study of 14 isozyme systems in 63 plants of napier grass (*Pennisetum purpureum*) did not show qualitative variation on any Loci (Shenoy and Vasil, 1992).

Isozyme analysis can also explain the genetic basis of certain somaclonal variations. If the variation is caused by a loss of a large portion of a chromosome or it involves the induction or removal of a large number of genes, it is expected that it will result in loss or addition of isozyme bands relative to standard phenotype. In contrast, if only a single gene is altered, it may not reflect in isozyme pattern.

Changes in isozyme pattern in somaclonal variants has been reported in celery (Orton, 1983) and wheat (Davies *et al.*, 1986). Modification of wheat B-amylase phenotype with additional activity bands, where the meiotic and mitotic chromosomal configurations were normal have been reported by Ryan and Scowcroft (1987).

2.7. *In vitro* regeneration studies in *Trifolium* species

Trifolium, being an important genus from fodder and pasture point of view, has attracted attention of various research workers for its genetic improvement through biotechnological tools. Many researchers have worked on important temperate *Trifolium* species such as *T. repens, T. pratense, T. subterraneum etc.* Sporadic efforts carried out through 1970s and 1980s got a boost after classical works of Phillips and Collins who devised a new basal media L2 for *T.pratense.* Only few reports are available on tropical and sub-tropical species such as *T. alexandrinum* and *T. resupinatum*. A brief account of *in vitro* organogenesis and embryogenesis studies carried out in different species are given below :

T. alexandrinum (Egyptian clover or Berseem clover) :

Plant were regenerated from hypocotyl and anther explants of berseem clover on MS medium containing various combination of plant growth regulators.

Mokhtarzadeh and Constantin (1978) cultured seedling hypocotyl section of berseem clover on MS medium containing various combinations of IAA, NAA,

2, 4-D, 2-ip, BA and KIN. Optimal callus induction occurred on a combination of 5.5 μM NAA and 7.5 μM KIN. Berseem clover was very sensitive to 2, 4-D. Callus was subsequently propagated on medium containing 11 μM NAA and 0.45 μM 2-ip. Cell suspension cultures were grown from callus inoculated in liquid medium containing 11 μM NAA and 0.9 μM 2-ip. Callus colonies were recorded from all suspensions inoculated on a solidified version of the same medium. Immature anthers excised from one plant also formed callus. Callus of all sources produced shoots when cultured on medium containing 2.7 μM NAA and 2.5 μM KIN. Certain other combinations of growth regulators yielded shoots less efficiently. Shoots were rooted on medium containing 5.7μM IAA and 0.44 μM BA. Plants were established in the green house.

Induction of shoots on MS medium with 0.5 mg/l each of NAA and KIN followed by induction of roots on MS medium with 1.0 mg/l of 6-BAP- suspension cultures in liquid. MS medium containing 2 mg/l of NAA and 0.2 mg/l of 2-ip provided filterable cell preparation with 45% viable cells. 4% of which gave rise to colonies within 3 weeks of transfer to agar plates. Shoot development was observed when callus from the colonies was cultured on MS medium with 0.5 mg/l of NAA and KIN. Twenty per cent of uncontaminated anthers from a single plant cultured on MS medium containing 1.0, 0.1 and 0.01 mg/l of NAA, 2, 4-D and 2-ip, respectively produced callus which could be maintained and from which plants could be regenerated by culturing on MS medium with 0.5 mg/l each of NAA and KIN. Preliminary results indicate that cells of root tips from hypocotyle and anther derived callus have the expected diploid and haploid number of chromosomes (2n= 16).

Barakat (1990) screened a range of MS based media for their ability to induce callus from root, hypocotyl and cotyledon explants of four *T. alexandrinum* cultivars. The MS medium containing 2.0 mg/l NAA and 0.5 mg/l BAP (MSP-1) was the one which gave optimum callus (Friable and fast growing) induction for all sources of tissues. However, callus growth was minimal on MS medium containing 0.05 mg/l NAA and 0.5 mg/l BAP (MSD-4).

MS medium containing 2.0 mg/l NAA and 0.5 mg/l BAP was highly effective in producing green nodules from the different callus types of all cultivars. Shoot regeneration was observed after 2 subculture on same media (MSP-1). Root formation was induced when regenerated shoot tips (1.2 cm) were transferred to MS based agar medium with 2.0 mg//L NAA, 0.03 mg/L Kinetin and 0.001 mg/L folic acid. Variation between cultivars has been observed mainly as effects of genotype on the regeneration of plants from callus (Barakat, 1990).

T. medium (Zigzag clover) : Petiole segments from two strains (CZO and Beaver lodge) of zigzag clover *(T. medium)* were cultured on L_2 or in SL_2 medium. Shoots were regenerated *via* organogenesis as well as somatic embryogenesis from petiole segments of both strains. Direct shoot regeneration was noticed as early as eight days after the initiation of cultures. Regenerated plants have normal morphological characteristics (Choo,1988). In an earlier study, no regeneration was obtained from callus culture (Parrott and Collins, 1982).

T. pratense (Red clover) : Initial attempts to generate callus from red clover tissue and plants from callus were disappointing. Niizeki and Kita (1973) cultured 99 anthers from two cultivars on the basal media of Miller (1961) and Bourgin and Nitsch (1967) modified with various combinations of IAA, NAA, 2, 4-D, BA and GA. Callus formation occurred only in a single instance on Miller medium containing 17 μM IAA and 6.6 μM BA. However, no morphogenetic development was observed. Microscopic evaluation indicated that the callus arose from somatic tissue.

Ranga Rao (1976) initiated callus from root, stem and leaf tissues of one cultivar and two breeding lines of red clover cultured on a basal medium modified from Miller (1961) and supplemented with 9 μM 2, 4-D and 10 μM KIN. Callus suspensions were subsequently cultured on liquid medium free of NO_3^-, 2,4-D and KIN and inoculated with a symbiotic strain of *Rhizobium trifolii*. In 6-8 weeks, 75% of the infected cultures produced roots regardless of the origin of the tissue. Un-inoculated tissues did not undergo morphogenesis. It was concluded that hormones produced and released by the *Rhizobium* in infected cultures were

responsible for root formation by the red clover callus.

Various combinations of 2, 4-D, IAA, NAA, KIN and corn milk were evaluated using a basal medium composed of the major salts of Gautheret (1955), minor salts modified from Burkholder and Nickell (1949) and organic components modified from Gautheret (1955) callus growth was obtained using 9 mM 2, 4-D and 15% corn milk. Very little growth occurred without the addition of corn milk. This suggests that undefined nutritional factors were missing from the basal medium (Zakrzewski and Zakrzewski,1976)

Ahloowalia (1976) cultured seeds of one tetraploid cultivar on MS medium supplemented with 7.5 μM 2, 4-D, 37 μM IAA, and 10 μM KIN. Callus was produced from 63% of the seeds within 6 weeks. Somatic embryos at the globular, heart, and torpedo stages were recovered from callus cultured on half strength MS medium free of growth regulators. No plants were regenerated.

Phillips and Collins (1979) investigated the growth of callus from seedling of five cultivars. Initial experiments evaluating the SH, B5 and MS basal media containing 2, 4-D and KIN were not very productive. Visual ratings of various combinations of NAA, IAA, CPA, 2, 4-D, PIC, KIN, 2-ip and BA were carried out. The combination of 0.25 μM PIC and 0.44 μM BA was optimal for callus initiation and cell proliferation. Since other basal media were deemed unsatisfactory, an improved basal medium designated L_2 was experimentally developed (Phillips and Collins 1979). The chemical forms and concentrations of individual components were visually rated. Major and minor inorganic elements and organic compounds were evaluated in that order. The final composition of the L_2 medium contained a major salts formulation similar to that of the MS medium, with a reduction in the concentration of NH_4^+ and increased concentrations of PO_4^{--}, K^+, Mg^{++} and Ca^{++}. The minor salts formulation was similar to that of the SH media with adjustments in the concentration of several salts. The organic formulation (Vitamins and Sucrose) was similar to that of Linsmaier and Skoog (1965) media with increased concentrtion of thiamine and myo-inositol. The addition of nicotinic acid was inhibitory to the callus growth. The L_2 medium was found to be more broadly supportive of red clover genotypes in culture than other

basal media. In tests with alfalfa (*Medicago sativa* L.) and soybean (*Glycine max* (L.) Merr.) The L_2 inorganic formulation was statistically shown to be superior to the MS, SH and Miller formulations, confirming that the L_2 medium was more broadly supportive of certain legumes in culture. The frequency of regeneration was dependent on the genotype and explant, with callus tissues derived from meristem having a higher regeneration capability than those from non meristematic regions (Phillips and Collins, 1979)

Phillips and Collins (1979) obtained vigorous callus from both mature and immature vegetative and reproductive explant sources on the L2 medium. Plants were regenerated from callus cultures. The frequency of plant regeneration was depended on the origin of the tissue and the source cultivars.

Cheyne and Dale (1980) obtained regeneration from shoot tip explant of one diploid cultivars and one tetraploid cultivar. B5 or Blaydes basal media supplemented with 1.2 μM IAA and 0.9 μ M 2-ip yielded about 67% plant regeneration frequencies. Bhojwani *et al.*, (1984) could not obtain organogenesis on MS supplemented with 2,4 D or BAP or 2-ip in various combinations with or without CH.

T. subterraneum (Subterranean clover) :

Earlier study of Graham (1968) failed to get any organogesis. He cultured seeds of subterranean clover on modified MS medium (Linsmaier and Skoog, 1965, Supplemented with 4 μM nicotinic acid) containing 0.2 μM KIN and 1 μM 2, 4-D to induce callus formation. Callus was maintained on the same medium. Cell suspension cultures were grown on a liquid version of the same medium. Cultures were inoculated with *Rhizobium* which did not interfere with growth. No morphogenetic development was observed.

Regeneration of *T. subterranean* was achieved by both shoot organogenesis and somatic embryogenesis. Regeneration in subterranean clover was obtained by culturing seedling hypocotyl on L2 medium. Shoot bud development was clearly visible on hypocotyl explants after three to ten days in L2 medium (i) shoot with trifoliate leaves were than formed often one month in culture. These shoots

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developed from the hypocotyl region whereas the radicle region showed no further development. Histological studies revealed that shoots arose *de novo* and did not originate from pre-existing meristems. Shoot derived via organogenesis produced roots within two weeks of transfer to RL medium and the regenerated plants had a normal phenotypic appearance. Hypocotyl explants showed no shoot but development in the absence of added growth regulators (L2 medium) (Heath *et al.,* 1993).

In the second regeneration protocol, Shoot apices taken from 10 days old seedlings of the cultivar Dalkeith were induced to form calli on L2 basal medium supplemented with the auxin picloram (21 μM) and maltose (4%) as the sugar source. Somatic embryos appeared spontaneously on the calli. A majority of embryos had a well- defined root pole, two cotyledon and were capable of germination, albeit at a low frequency. Regenerated plants obtained from both protocols appeared phenotypically normal.

T. repens (White clover) :

Pelletier and Pelletier (1971) cultured cotyledons of one white clover cultivar. Callus was grown on a medium composed of MS major salts, Heller minor salts, B vitamins, agar, 0.17 M glucose, 2,4-D and KIN. Callus was transferred to medium containing 0.5 μM NAA, 0.5 μM KIN and 10% CW after 6 weeks. Callus derived from a single cotyledon exhibited organization. They both chromosomal and morphological variation in the regenerates. However, in later work, Gresshoff (1980) was unable to find any chromosomal differences in morphologically variant regenerants, even in plants obtained up to 2 years after culture initiation.

Rupert and Seo (1977) regenerated plants from callus derived from embryos following sexual hybridization of white clover with *T. ambigum*, the callus remaining totipotent for up to 3 years in culture. Maheswaren and Williams (1984) cultured immature embryos of white clover, and obtained a clone of axenic plantlets through primary and secondary embryogenesis.

Bhojwani *et al.,* (1984) and White (1983, 1984) emphasized the importance of selection for regenerating genotypes in white clover, and isolated two plants, TR-20 and WR8 , with high regeneration capacity. Bhojwani *et al.,* (1984) could obtain plantlets on MS media supplemented with 2-ip (0.5mg/L) and IAA (0.1 mg/L).

Parrott and Collins (1982) have successfully obtained callus and cell suspension cultures from white clover using the methods and media developed by Phillips and Collins (1979, 1980) for red clover. Callus derived from 20% of the tested genotypes produced roots.

Gresshoff (1980) cultured seeds and seedling section of one cultivar on B5 or MS basal media supplemented with 0.5 μM KIN and either 4 or 10 μM 2,4-D. Callus induction was complete on the higher concentration of 2, 4-D.

Richard and Rupert (1980) found that a modified MS medium containing 2.9 M IAA and 4.5 M 2-ip encouraged shoot multiplication from cultured embryos.

When primary somatic embryoids derived from immature sexual embryos of *T. repens* are sub cultured in a medium containing 1-2 mg/l BAP. Secondary embryoids arise directly by multicellular proliferation of superficial cells on the lower surface of the axis and cotyledonds. The secondary embryoids develop into small leafy shoots on the induction media and are easily detached for rooting on hormone free medium. Specialization of walls determining embryoids from tissue apparently contributes to this case of separation. Using this secondary subculture technique, it is possible to obtain a clone in the order of 100 rooted plantlets from one immature sexual embryos within 10-14 weeks from pollination. (Maheswaran and Williams,1986)

Cotyledons from immature embryos of *T. repens* var. Oscola were exposed to 2, 4-D or NAA to induce somatic embryogenesis. NAA at 10 or 20 mg/l was very inefficient at stimulating embryogenesis, while concentrations of 30 or 40 mg/l resulted in death of the explant tissue. Continuous exposure of cotyledons to 40 mg/l, 2, 4-D resulted in somatic embryos which are arrested at the globular stage. A 10 day exposure time to 2, 4-D at the same concentration led to formation

of somatic embryos, most of which had poorly developed cotyledons. Almost 10% of the somatic embryos converted into plants following transfer to medium devoid of growth regulators. Attempts to improve morphology of somatic embryos by using shorter exposure times to 2, 4-D at 40 mg/l or by maintaining the 10 day exposure time while varying the concentration of 2, 4-D were not successful. (Parott, 1991)

Somatic embryos were obtained from immature cotyledons of white clover cultivar Oscola placed into EC6 basal medium containing 40 mg/l of 2, 4-D and 6% sucrose. Repeated sub-culture of white clover somatic embryos on EC6 basal medium containing 6% sucrose with 2,4-D at 20 or 40 mg/l effectively maintains repetitive embryogenesis. Medium containing MS salts with 6% maltose as the carbohydrate source was the most efficient for plant recovery. (Weissinger and Parott, 1993).

In white clover most prolific and rapid plant regeneration occurred on MS based media containing NAA and BAP, while other phytohormone combinations, 2, 4-D or picloram with kinetin 2-ip resulted in either extensive callus formation or distorted shoot development (White and Voisey, 1994). They conducted a series of experiments to screen the potential of roots, hypocotyl and cotyledons from seedlings to regenerate. Results indicated that cotyledon from 3 day old seedlings were most responsive. Regeneration was obtained from the cotyledon of white clover using MS basal medium supplemented with varying concentrations of NAA and BAP. The highest shoot regeneration frequency (an average of 20 shoots per cotyledon) was obtained using MS medium containing 1.0 mg/l. BAP and 0.05 mg/l NAA. A similar regeneration frequency was obtained from cotyledon explants taken from eight different white clover cultivars and no genotypic effect was observed.

Other Clovers

Trifoliums of less economic importance than red and white clovers have also been cultured, and regeneration described for *T. incarnatum* (Crimson clover; Beach and Smith, 1979). Less attention has been given to wild species, although Parrott and Collins (1982) reported somatic embryogenesis from seedling explants

of *T. rubens*, while Bhojwani *et al.*. (1984) could not obtain organogenesis in *T. arvense*.

Schenk and Hildebrandt (1972) obtained callus growth from seedlings of Alsike clover cultured on SH medium containing 2.2 μM 2, 4-D+ 11 μM CPA and 0.5 μM KIN. No morphogenetic development was reported. Beach and Smith (1979) obtained callus from Crimson clover in the same manner as from red clover.

Parrott and Collins (1982) cultured large hop clover (*T. campestre*) subterranean clover, crimson clover, *T. alpestre*, zigzag clover and *T. rubens* following procedures developed by Phillips and Collins (1979, 1980) and Collins and Phillips (1982) for red clover. Large hop clover failed to respond to shoot tip culture while rapid clonal propagation was achieved by with *T. alpestre,* Crimson clover, *T. rubens* and subterranean clover. The latter species multiplied most rapidly in shoot tip culture. Zigzag clover produced callus as well as shoots on the standard medium which was correlated by using one third the normal concentration of PIC as auxin. Subterranean clover rooted prolifically on the standard rooting medium. Rooting frequencies of the other species were more efficient using only water. Callus of subterranean clover, large hop clover, and *T. alpestre* did not grow well, exhibiting varying degrees of necrosis. The L2 medium was suboptimal for these species. About 20% of the *T. alpestre* genotypes rooted before the callus turned brown. All callus of subterranean clover rooted. One callus sector of large hop clover survived and proliferated slowly. Zigzag clover and *T. rubens* produced callus which grow more vigorously than that of red clover, while callus of crimson clover grow more slowly. Some crimson clover callus developed buds and roots.

MATERIALS
&
METHODS

3. Material and Methods

The present work was carried out to see the response of genotype, explants and media combinations for *in vitro* regeneration in some species of *Trifolium*. Different materials used and methods applied in the present study are described below.

3.1. Materials

3.1.1. Procurement of genotypes

The study involved six genotypes of *T. alexandrinum* and six species of *Trifolium*. The genotypes were procured from the '*Trifolium* genetic improvement project' of IGFRI, Jhansi and used in the present study

3.1.2. Genotypes of different *Trifolium* species : One genotype each of six different *Trifolium* species was used in this study.

Sr. No.	Species	Accession number / genotype
1	*T. resupinatum*	SH 97 – 49
2	*T. subterraneum*	IG 96 – 112
3	*T. repens*	EC 400986
4	*T. hybridum*	EC 401702
5	*T. apertum*	EC 401712
6	*T. glomeratum*	EC 401700

3.1.3. Genotypes of *Trifolium alexandrinum* :

Six genotypes of *T.alexandrinum* were used in present study, of which 3 were diploid ($2n = 2x = 16$) and 3 were tetraploid ($2n=4x=32$).

(A) Diploid genotypes

       FAO-1 - (JHB 97-1) an advanced breeding line

       JHB 146 - (Bundel Berseem-2), a high yielding released variety.

       BL-142- An advanced breeding line

(B) Tetraploid genotypes

       1-90 P - (JHTB 96-4) an induced autotetraploid line

       9-90-N- an induced autotetraploid line

       3-90-H - (JHTB 97-3) an induced autotetraploid line

3.2 Methods

3.2.1 *In vitro* studies in different *Trifolium* species :

Raising of plants :   Healthy seeds of respective genotypes were surface sterilized by immersing in 0.1% $HgCl_2$ for 1-1.5 minutes. This was followed by 4-6 washings in sterile distilled water. Seeds were then germinated on MS media containing 3% sucrose and solidified with agar (0.7%) without any growth regulators. These were incubated in dark at 25 ± 2^0C till germination. After germination cool fluorescent light was provided for 8-10 hours per day. Seedlings attained the height of 2.5-3.5 cm 20-25 days after germination.

Explants : Explants were taken from 20-30 days old healthy plants grown under aseptic conditions. Different plant parts were excised and 0.3 - 0.4 cm long pieces were cultured on the callusing media. The plant parts used as explants were leaf, petiole, hypocotyl, cotyledon, collar and root.

Media: Different combinations of inorganic media with varying levels of hormonal concentrations were used at different stages of growth (Table 3.2).

Preparation of media

Basal media - Three basal media (MS, L2 and RL)were used in present study. The composition is given in Table 3.1.

MS media : It was used for germination of seeds. The inorganic salts (Murashige and Skoog, 1962) were mixed in double distilled water supplemented with 0. 3% sucrose and solidified by adding agar 0.7%.

L2 media : This media was used for callus induction and differentiation. Inorganic salts (Phillips and Collins, 1984) were mixed in double distilled water (Table 3.1). Growth regulators were added in different concentrations for various purposes.

RL media : This media was used for root induction in shoots. Inorganic salts and growth hormones were added as per Phillips and Collins (1984) (Table 3.1).

Preparation of basal media - Different basal media were prepared by mixing appropriate combinations of inorganic salts as mentioned in table 3.1. The salts were dissolved by adding one salt at a time. Precipitation was avoided by dissolving the inorganic Nitrogen source first. Growth hormones were added in different combinations for specific objectives before the addition of agar. pH of the media was adjusted to 5.8 using 1N NaOH. After the addition of agar and sucrose the medium was poured into test tubes and plugged with non-absorbent cotton wrapped in muslin cloth. The medium was autoclaved for 25 minutes at 15 Lbs/sq. inch pressure. Autoclaved medium was allowed to cool quickly and kept in dark. After the solidification at room temperature the medium was used for various purposes such as callus induction, proliferation and regeneration.

Preparation of culture media : Different culture media were prepared by varying the level of hormones to these basal media. Different media used for specific purpose are given in table 3.2.

Culture conditions : The cultures were maintained at $25^0 \pm 2^0 C$. The seeds were germinated in total dark in culture room at 25 ± 2^0 C temperature. After germination, the seedling were provided with 8-10 hrs. cool fluorescent light during the stage of shoot and root development. The explants were excised from the seedlings and cultured on the medium. Initially the explants were kept in dark for callus induction but after 5 days of inoculation the cultures were provided with a day light cycle of 8/10 hours. After shoot emergence, the light period was increased to 10-12 hrs for shoot multiplication and growth.

Callus induction : Explants from healthy seedlings were inoculated aseptically in the culture tubes containing callus induction media. The experiments were carried out in 5 sets. Each set had 3 culture tubes with 2 explants in each tube. A total of 30 explants were cultured per explant media genotype combination. In a few cases, the number of explants cultured were less because of shortage of healthy explant pieces or due to contamination. The contaminated tubes were discarded and not taken into consideration while tabulating the data.

The periodical observation was taken on nature, colour and growth rate of callus. After 30-35 days, the calli were split and sub-cultured in 10-15 tubes to either shoot inducing media or Somatic Embryogenesis Inducing Media (SEIM). A part of the non – differentiating callus was also sub-cultured in the same callus inducing media to observe any differentiation at the later stage. These calli were also used for cytological and isozymic studies.

Induction of Shoots : Induced callus after 35 days of explant inoculation were sub-cultured in different shoot inducing media. A small part of the calli were inoculated aseptically in 10-15 tubes in different genotype, media explant combinations.

Periodic observations were taken and any differentiation was noted. In cases, where shoots were induced, the shoots were multiplied by one more round of sub-culturing in the same culture media. In some cases, root induction was also observed in this media itself and such plantlets were transferred to field after hardening

Induction of roots : The shoots were split and sub-cultured again on root induction media (RL). The lower part of the tubes were covered with black paper to facilitate the root induction. Cool fluorescent light for 10 hours per day was provided at 25 ± 2 C. Repeated sub-culturing of shoots was done in cases where no root induction was observed.

Hardening and field transfer : Complete plantlets with root and shoot were hardened and transferred to field. The tubes were taken out of culture room and kept at room temperature for 2-3 days. The plantlets were then taken out of tube and washed carefully to remove the agar media. Extra precaution was taken at this stage so as to cause minimum damage to root or shoots.

Nodulation : Old plants of the respective species were taken from the field and nodules were removed. These nodules were macerated in distilled water and the root portion of regenerated plants was dipped for overnight in this solution. The plants after overnight treatment were transferred to pots having soil : sand ratio of 1:1. The process helped in induction of nodules in the regenerated plants in 6-7 days. The pots were kept every day

for 3-4 hours in the nursery for 6-7 days. Extra care was taken to avoid desiccation of young leaves. This was done by wrapping cellophane paper across the plant with sufficient holes for air transfer. After 6-7 days the plants were transferred to field. Artificial shade condition was provided for initial 7 days to acclimatize the plant to new environment.

Somatic embryogenesis : The callus obtained form different genotype-media-explant combinations were split and inoculated in Somatic Embryogenesis Induction Media (SEIM). In each combination set 10-15 tubes were cultured. The formation of embryoids was noted

***In-vitro* response study in different genotypes of *T. alexandrinum* :** Six genotypes, three diploid (2n=16) and three autotetraploid were used in the present study. Four explants namely, leaf, petiole, hypocotyl and collar were used in the study. The process of callus, shoot, root induction, hardening and field transfer were same as described above.

Statistical analysis : The data obtained for callus induction in different genotype-media-explant combinations were grouped. Each set having 3 culture tubes with 2 explants in each tubes was taken as one set and mean $\pm$ standard deviation was calculated based on 5 such sets.

Analysis of variance test or 'F' test was done to analyze the results obtained. F test is developed by R. A. Fisher and is done to test whether two or more sample means have been obtained from populations with the same parametric mean with respect to a given variable. Alternatively, it can be said that these mean differ from each other to such an extent that it may be assumed that they are sampled from different populations (Sokal and Rohlf, 1969). F value *i.e.* the ratio of variance is the ratio between mean sum of squares among group and error.

The observed value of F was compared against the table critical value of F distribution against the degree of freedom of denominator and numerator. If the observed value was found lower than the table value at 5% level, the difference was said to be non-significant. If the observed value was more than the table value at 5% or 1%

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level, the difference was termed as significant or highly significant respectively. Higher order factorial ANOVA (2-way and three way) was carried out to compare the effect of interaction of various factors.

3.2.2. Biochemical characterization of the regenerant and calli :

The regenerant as well as calli of different ages were characterized on the basis of their isozyme patterns for different enzymes.

Isozyme pattern of mother plant, regenerant, callus and regenerant at different age and stages were compared for the isozyme variations. Isozyme systems used in the present study include : Peroxidase (Per, E.C.1.11.1.7), Esterase (Est, E.C.3.1.1.2), Super-oxide dismutase (SOD, E.C. 1.15.1.1), Acid phosphatase (Acp, E.C.3.1.3.2) and Glutamate oxalo-acetate transaminase (GOT or AAT, E.C.2.6.1.1.).

Horizontal starch gel electrophoresis technique of Smithies (1955) with discontinuous buffer system of Poulik (1957) was used for studying the enzyme systems, because it has a better resolving power than other techniques (Brewer and Singh, 1972) which results from the gradation of molecular sieving effect of starch gel matrix besides electrophoretic separation.

Preparation of enzyme extract : The crude extract from the leaves was prepared by homogenising 1g of sample with 0.3 ml of tris buffer (pH 8.65), in a pre-chilled pestle and mortar. The crude extract was filtered through muslin cloth and the filtrate was stored in the deep fridge ($-20\ ^0$C) in different vials. Each vial was thawed only once just before use. Similarly crude extracts from calli were also prepared by homogenising 1 g of calli with 0.3 ml of tris buffer.

Preparation of buffers :

Gel buffer : Tris citrate buffer (pH 8.65) was prepared by dissolving 9.207 g tris and 1.051 gm of citric acid in 1 liter of distilled water.

Bridge buffer : 0.3 M sodium borate buffer (pH 8.65) was prepared by dissolving 18.552 g boric acid and 4 g of sodium hydroxide in distilled water so as to make final

volume up to 1000 ml.

Acetate buffer : 0.2 M acetate buffer (pH 5) was prepared by dissolving 27.216 g of sodium acetate trihydrate + 2.6 ml of acetic acid in distilled water so as to make final volume of 1000 ml.

Phosphate buffer (pH - 6.5) :

(a) Mono basic - 31.2 g of sodium dihydrogen orthophosphate was dissolved in double distilled water and final volume was made up to 1000 ml.

(b) Dibasic- 28.4 g of disodium hydrogen orthophosphate was dissolved in double distilled water and final volume was made up to 1000 ml.

AAT buffer (pH- 7.4) :

Following compounds were added to 100 ml of distilled water.

μ ketoglutaric acid - 37 mg

L Aspartic acid - 134 mg

PVP-40 - 500 mg

EDTA, Na$_2$ salt – 50 mg

Sodium phosphate dibasic - 1.45 g

Preparation of the gel : As per the technique standardized in the laboratory, 14% starch was used in the experiments. In a conical flask, 22.4 g of hydrolyzed potato starch and 160 ml gel buffer were mixed and heated with vigorous shakings, till it became less viscous and more translucent. The cooked starch was immediately poured on to a glass plate (12 cm x 12 cm) having 4 tires of glass strips, pasted on it. The tray was covered with a clean glass plate avoiding any air bubbles. The gel was allowed to set by keeping at room temperature for 5-6 hours in undisturbed condition.

Application of samples : The glass plate cover from gel was removed with the help of a blade. Slots were made in the middle of the gel for peroxidase and proximally for the other 4 enzymes with the help of the stick cutter. The samples to be analyzed were soaked on to filter paper wicks (Whatman no. 3) of size 5 mm x 8mm and inserted into the slots.

Electrophoresis : The gel plate was then placed in 'Genei' horizontal migration chamber. Bridge buffer was poured into the buffer chambers so that electrodes were completely dipped. The plate was connected with the buffer chamber with the help of filter papers (Whatman no. 1), which was earlier saturated with buffer.

Electrophoresis was carried out at constant current of 24 mA for first 30 minutes followed with constant current of 34 mA. The migration chamber was placed in a refrigerator to avoid overheating of the gel. The power supply was cut off when the buffer front reached the anodal end. The gel plate was taken out and the glass strips were removed from the sides of the glass plate. Each gel was sliced thrice horizontally, with the help of a copper wire, to obtain four slices of gel. The upper slice was rejected and lower ones were used for staining.

Staining of the gels : The gels were stained following the method given by Veech (1969) for peroxidase. For esterase, GOT, SOD and ACP staining was done as per method of Wendel and Weeden (1989) with slight modifications.

Peroxidase : Benzidine (100 mg) was dissolved by heating in 100 ml of 0.2 M acetate buffer (pH 5). 2 ml of 3% hydrogen peroxide was added at the time of incubation of the gels. After 10 minutes of incubation, blue band appeared which turned brown later.

Esterase : Gels were incubated in 100 ml of 0.1 m phosphate buffer (pH 6.5) containing 32.5 mg of 1- napthyl acetate in 1 ml acetone and 50 mg of fast blue RR. Gels were incubated at room temperature for 60 minutes. The esterase isozymes activity sites appeared as reddish brown to blackish bands on the gel.

Acid phosphatase The gels were incubated in 100 ml of acetate buffer (pH 5.6) containing 100 mg of Na-1-napthyl phosphate, 100 mg $MgCl_2$ and 100 mg of fast blue RR at 37^0C in the dark until desired staining intensity has occurred.

Aspartate Amino transferase (AAT) or Glutamate Oxalo-acetate Transaminase (GOT). The gels were incubated in 100 ml of AAT substrate solution containing 100 mg fast blue BB salt. The gels were incubated in dark at room temperature, until blue bands appeared.

Superoxide Dismutase : The gels were incubated in 100 ml of Tris-HCl buffer (pH-8.65) containing Riboflavin (4 mg) EDTA (2mg) and NBT (20 mg). The gel was incubated in dark for 30 minutes and then exposed to intense light for 1.5 hours.

Preparation of zymogram : The different protein bands were drawn on graph sheet at 1:1 ratio. The point of origin and front was marked in order to see the relative mobility of the bands. Selected gel plates were also photographed.

Scoring and nomenclature of bands : The bands were scored from starting point *i.e.* the well where the samples were loaded. The slowest band was treated as first band and the bands were numbered on the basis of pooled zymogram prepared for that particular species.

Similarity matrix analysis and cluster based on isozyme bands : A binary data matrix reflecting the presence or absence of specific isozyme band was generated. Only unambiguously scored bands were used in the matrix. Binary data matrix using isozyme banding pattern data has also been followed by other workers (Pisupati, 1999).

The genetic similarities (GS) between line i and j were estimated using the formula of Dice (1945) $Gs_{ij} = 2N_{ij} (N_i + N_j)$,

Where, N_{ij} is the number of common bands between i and j and N_i is the number of bands in i and N_j is the number of band in j respectively.

A dendrogram was generated using the unweighted-pair-group method average (UPGMA) clustering procedure. All computations were done using the computer software NTSYS-PC version 1.60.

3.2.3. Cytological study of calli of different ages :

Proliferating calli of different ages were selected for the cytological study. The calli were pre-treated in chilled saturated aqueous solution of para- dichlorobenzene (pDB) at 7.00 A.M. for 7 hrs followed by fixation in 1:3 aceto-alchohol for overnight. It was then hydrolyzed in 1N HCl for 8 minutes at 60^0C. The slides were prepared following squash technique after staining in 2% aceto-carmine for 2 hours. Slides were observed under microscope for numerical variation in chromosome number. Photographs were taken using Nikon labophot 2 Camera at different magnification.

Preparation of reagents :

2% aceto-carmine : Two gram of Carmine powder was added slowly to 100 ml of boiling 45% acetic acid and kept at simmering condition for 30 minutes. This solution was then cooled, filtered using Whatman no.1 filter paper and stored in air-tight glass bottle.

Fixative : To one part of glacial acetic acid, 3 parts of absolute ethanol was added and mixed well. Traces of $FeCl_3$ were also added to this fixative for better staining.

Normal HCl : To 91.67 ml of distilled water, 8.33 ml concentrated HCl was added slowly, thoroughly mixed and stored.

Table 3.1. Composition of L2, MS and RL basal media used in present study

S N	Components	L2 basal	MS basal	RL basal
1	KNO_3	20.8 mM	18.8 mM	10.4 mM
2	NH_4NO_3	12.5 mM	20.6 mM	6.25 mM
3	KH_2PO_4	2.34 mM	1.25 mM	2.34 mM
4	$MgSO_4. 7H_2O$	1.8 mM	1.5 mM	0.9 mM
5	$CaCl_2. 2H_2O$	4.1 mM	3.0 mM	2.0 mM
6	NaH_2PO_4	0.6 mM	-	0.3 mM
7	$FeSO_4.EDTA. 7 H_2O$	90 μM	100 μM	90 μM
8	$Na_2 EDTA. 2H_2O$	-	100 μM	-
9	$MnSO_4. 4 H_2O$	90 μM	100.0 μM	45 μM
10	H_3BO_3	82 μM	100.0 μM	41 μM
11	$ZnSO_4. 7H_2O$	18 μM	30.0 μM	9 μM
12	KI	6 μM	5.0 μM	3 μM
13	$Na_2MoO_4. 2H_2O$	1.7 μM	1.03 μM	0.85 μM
14	$CoCl_2. 6H_2O$	0.42 μM	0.105 μM	0.21 μM
15	$CuSO_4. 5 H_2O$	0.4 μM	0.1 μM	0.2 μM
16	Myo-inositol	1.4 mM	100 mg/L	0.7 mM
17	Thiamine HCl	6 μM	0.1 mg/L	3.0 μM
18	Pyridoxine HCl	2.4 μM	0.5 mg/L	1.2 μM
19	Nicotinic acid	-	0.5 mg/L	8.5 μM
20	3-Aminopyridine	-	-	24 μM
21	Sucrose	73 mM	87.6 mM	44 μM
22	Agar	0.8 %	0.7%	0.65 %
	pH	5.8	5.8	5.8

Table 3.2 Composition of various media used in the present study

Media	Basal media	Auxin (mg/L)		Cytokinin (mg/L)	Auxin : Cytokinin
		NAA	Picloram	BAP	
Seedling raising media					
MS	MS	-	-	-	-
Callus inducing media					
A	L2	0.05	-	0.10	0.5 : 1
B	L2	1.25	-	0.25	5 : 1
C	L2	2.50	-	0.50	5 : 1
D	L2	5.00	-	1.00	5 : 1
A-1	L2	-	-	0.10	-
L	L2	-	0.06	0.10	0.6 : 1
P	L2	-	0.60	1.00	0.6 : 1
Shoot inducing media					
E	L2	0.0008	-	0.150	0.0053 : 1
F	L2	0.02	-	0.375	0.053 : 1
G	L2	0.04	-	0.750	0.053 : 1
H	L2	0.08	-	1.500	0.053 : 1
Somatic embryogeneis media					
		2,4 – D		Adenine	
SEIM	L2	0.001		3.225	0.0003 : 1
Root inducing media					
		IAA			
RL	RL	0.21	-	-	-

Table 3.3 Details of important chemicals used in the study

Chemical	Make	Catalogue number	Molecular weight
1- Napthyl acetate	Loba Chemie	4785	186
Fast Blue RR	BDH	28377	
NBT	SRL	144928	817.65
Riboflavin	HiMedia	RM 181	376.37
EDTA	Hi media	RM 678	292.25
α ketoglutaric acid	Hi media,	RM-245	146.1
L Aspartic acid	Hi media	RM-083	33.10
PVP-40	SRL	164798	40,000
EDTA, Na_2 salt	Qualigens	18454101	372.24
Sodium phosphate dibasic	Loba Chemie	5972	141.96
Fast blue BB	Hi media	RM-814	
Sodium 1- Napthyl phosphate	Loba Chemie	5945	264.15
Tris	SRL	2044122	121.14
N-N-N-N- Tetramethyl ethylendiamine (TEMED)	SISCO	202788	116.21
Acrylamide	SISCO	014022	71.08
N-N Methylene Bis acrylamide	SISCO	134985	154.17
Ammonium Per Sulfate	SISCO	0148134	228.20
Citric acid	Loba		192.13
Boric acid	Loba	43118	61.83
NaOH	Loba	43052	
Sodium acetate trihydrate	Loba	46174	136.08
Glacial acetic acid	Qualigens	21057	60.05
Sodium dihydrogen orthophosphate	Hi Media	RM256	156.01
Disodium hydrogen Orthophosphate	Loba	45675	141.96
6-BAP	Hi Media	RM 787	
NAA	Hi media	RM575	186.21
Picloram	Sigma	P-5575	242.5
IAA	Hi Media	RM 384	175.19
Agar			

Plate 1. A view of diploid *T. alexandrinum* genotypes

    A. FAO-1

    B. JHB 146 (Bundel Berseem 2)

    C. BL 142

Plate : 1

BUNDEL BERSEEM- 2

DIPLOID BERSEEM
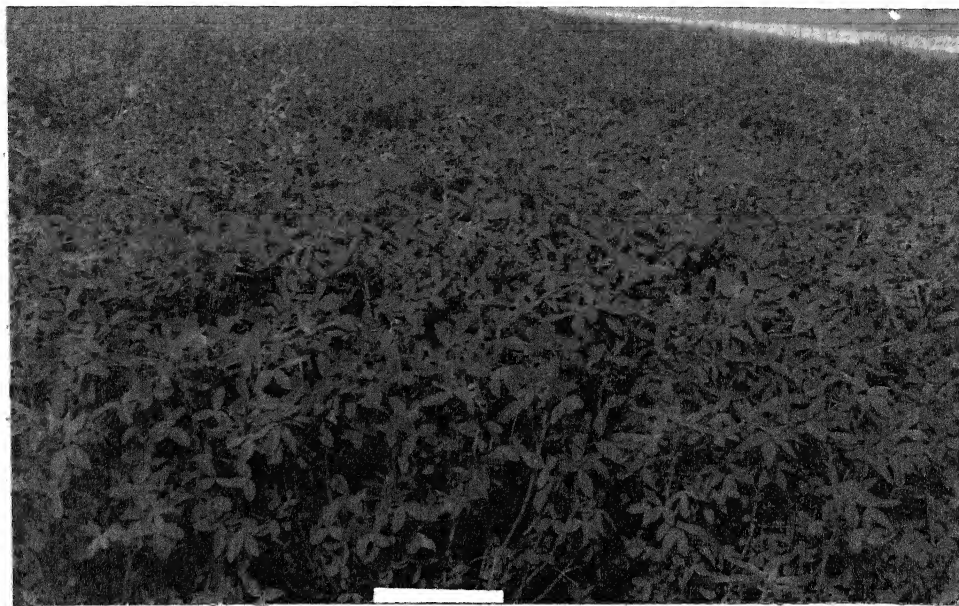
A

B
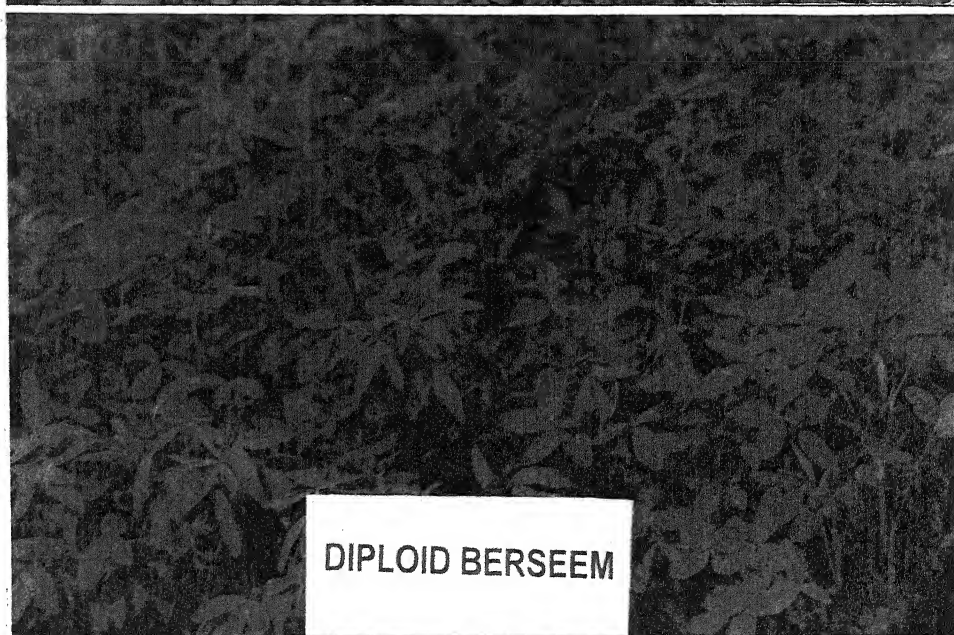
C

Plate 2.   A view of tetraploid *T. alexandrinum* genotypes

    A.   1-90 P (JHTB 96-4)

    B.   3-90 H (JHTB 97-3)

    C.   9-90 N

Plate : 2

JHTB 96-4

A

JHTB 97-3

B

TETRAPLOID BERSEEM
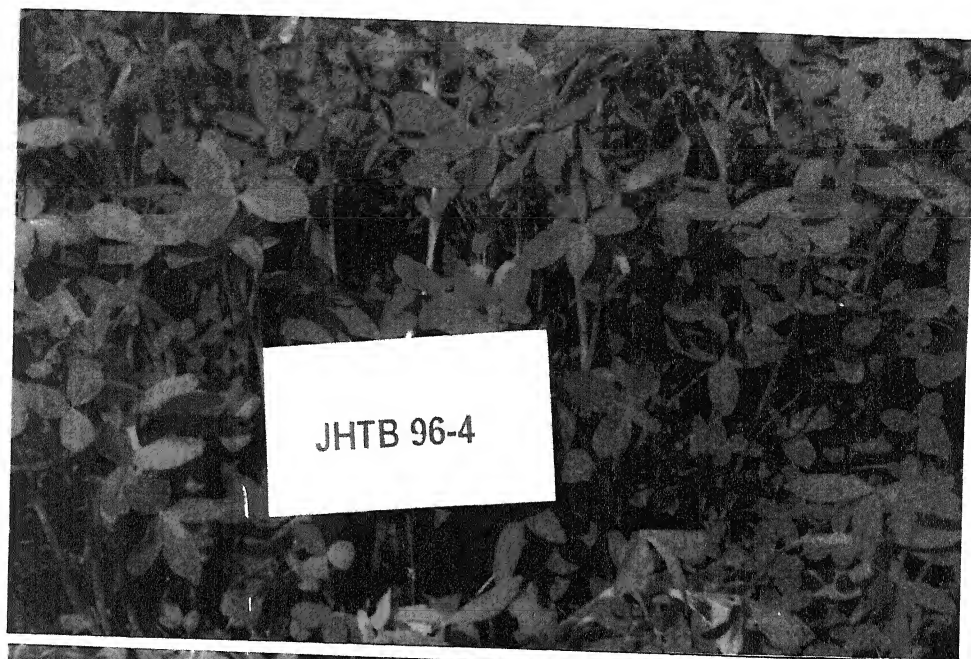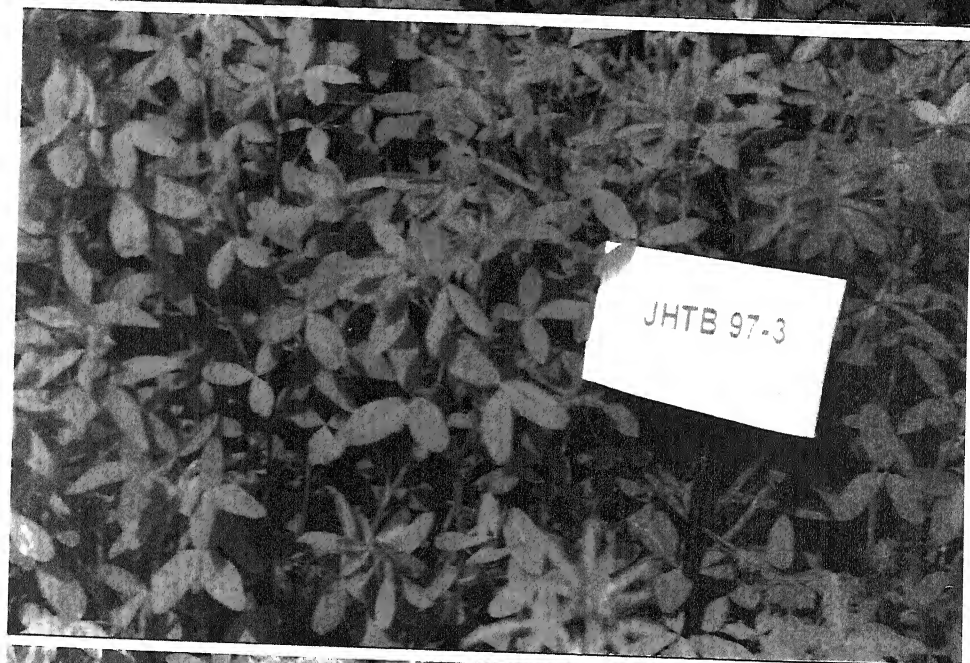
C

Plate 3.   A view of different *Trifolium* species

A. *T. apertum*   (EC 401712)

B. *T. repens*   (EC 400986)

C. *T. resupinatum*   (SH 97-49)

Plate : 3

A
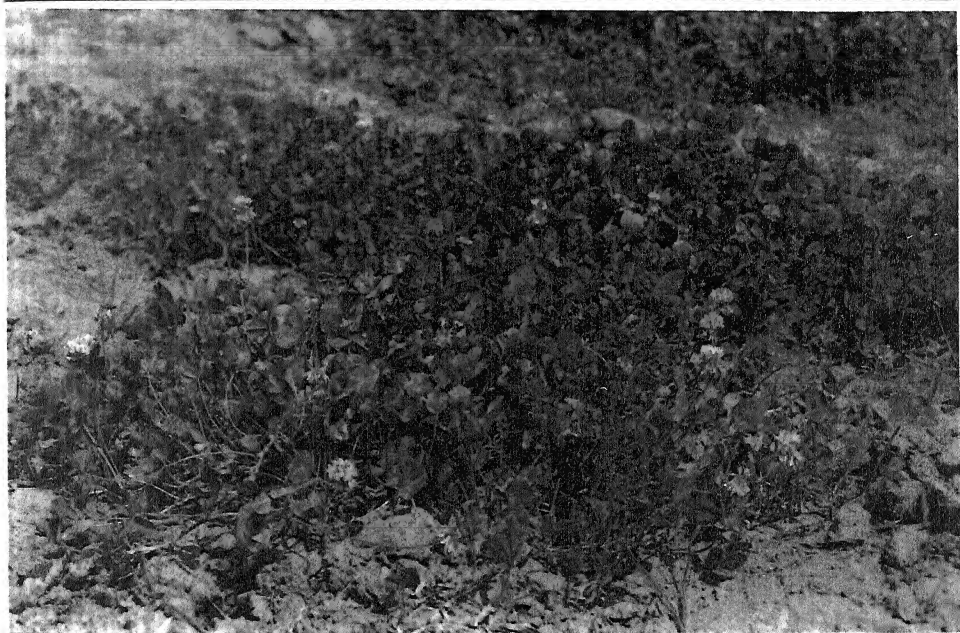
B

C

RESULTS

4. Results

In vitro response of various explant-media combinations were carried out in different *Trifolium* species and different genotypes of *T. alexandrinum*. Experiments were carried out to study the effects of interaction of various factors such as genotype, media, explants source, age of explants etc. The various parameters used for evaluation for such interaction includes callus induction frequency, nature and growth rate of callus, sub-culturing response towards organogenesis and embryogenesis.

4.1. *In – vitro* response of different *Trifolium* species :

4.1.1. *T. resupinatum*

Healthy seeds of *T. resupinatum* (genotype SH 97-49) were selected and allowed to germinate on MS medium, devoid of any hormone under aseptic conditions. The seeds germinated within 5-6 days of culture. About 80% seeds developed into 3.5 to 4.0 cm long seedlings in 21-23 days. At this stage, explants like hypocotyl, petiole, cotyledon, leaf, collar and root were excised and cultured on two media for callus induction and results are presented in Table 4.1.

Leaf : In 'A-1' media response of explants was poor. Only 16.7% explants resulted in callus induction. The callus was friable, yellow in colour and slow growing.

In 'A' media moderate response (30%) of callus induction was found. The callus was friable/ compact and slow growing. No chlorophyll pigmentation was observed.

Petiole : In 'A-1' media, 43% of explants developed into callus. The swelling of explants was observed within 4-6 days and friable callus was formed. The rate of growth was slow to medium. The calli failed to develop pigmentation and were yellow in colour.

The explants showed a better response (67%) of callus induction in 'A' media. The rate of growth of friable, yellow to hyaline calli was slow to medium as they reached a diameter of 4-5 mm only in 35 days.

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Table 4.1: Callusing response of *T. resupinatum* explants in different medium

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Leaf	A-1	30	16.7±20.41	Friable	Yellow	Slow
	A	30	30.0±18.26	Friable, compact	Yellow, pale green	Slow
Petiole	A-1	30	43.3±38.37	Friable	Yellow	Slow, medium
	A	30	66.7±20.48	Friable	Yellow, hyaline	Slow, medium
Cotyledon	A-1	30	50.0±11.79	Compact	Yellow, Green	Medium
	A	28	68.7±12.38	Compact, Nodular	Pale Green, Yellow	Medium, High
Hypocotyl	A-1	30	50.0±26.35	Friable	Yellow	Slow
	A	28	92.7±10.11	Friable	Yellow, Green	Medium, High
Collar	A-1	25	36.0±21.91	Friable, compact	Hyaline	Slow
	A	25	20.0±24.49	Compact	Yellow	Medium, slow
Root	A1	27	0	-	-	-
	A	26	100	Friable	Yellow, Pale Green	High

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Cotyledon- In 'A-1' media, 50% of the explants showed callusing. The callus of 4.5-5 mm diameter was formed in 25-27 days of culture. The callus formed was compact and yellow.

In the medium 'A' 68.7% explants formed callus. Callusing initiated in 12-14 days and reached the size of 6.5-7 mm in diameter in the next 10-11 days. Nature of the callus formed was compact and nodular yellow coloured but in few cases the callus was slightly green. The rate of growth was medium to high in different sets.

Hypocotyl- In 'A-1' media, the explants showed a good response (50%) of callus induction. The explants showed swelling in about 9-11 days and pale yellow, friable callus was initiated in about 17-19 days. The callus was slow growing and attained the size of 4-4.5 mm in diameter in 28-30 days.

In the medium 'A' almost all the explants responded positively for callus induction and proliferation. Swelling in the explants was induced in about 9-11 days and friable, green callus was induced in 13-14 days which after 35 days reached the size of 8-8.5 mm diameter, the callus growth was nodular with tuber like protuberances.

Collar : In 'A-1' media, moderate response (36%) of callus induction was noticed. The callus was friable/compact, slow growing and hyaline.

In 'A' media, the response of explants for callus induction was poor (20%). The callus was compact and slow to medium growth rate was observed in different sets. However, the colour was yellow and no green pigmentation was observed.

Root- In 'A-1" media, the root explants failed to show any positive response for callusing. But in 'A' media almost all the explants formed friable, yellow calli. Though the callus proliferated vigorously, it failed to develop pigmentation and remained hyaline to pale yellow in colour.

Organogenesis- The callus obtained from each explant media combination was split and cultured in 10 tubes each in shoot inducing medium 'E' (Table 4.2).

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Table 4.2 : Subculturing response in *T. resupinatum*

Explant source	Callus ing media	Response of callus to shooting media on sub-culture (%)				Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		NR	CP	Shooting	Rooti ng	NR	Shooting	Rooting		
Leaf	A-1	10.0	90.0	-	-					
	A	30.0	70.0	-	-					
Petiole	A-1	100.0	-	-	-					
	A	100.0	-	-	-					
Hypocotyl	A-1	80.0	20.0	-	-					
	A	50.0	30.0	20.0	-	37.5	62.5	37.5	37.5	25.0
Cotyledon	A-1	90.0	10.0	-	-					
	A	50.0	20.0	20.0	10.0	37.5	37.5	25.0	25.0	-
Collar	A-1	70.0	30.0	-	-					
	A	80.0	20.0	-	-					
Root	A	40.0	40.0	20.0	-	40.0	16.7	33.3	33.3	60.0

NR = No response   CP = Callus proliferation

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No positive response of sub-culturing was observed in the calli developed from leaf, petiole and collar in any of the two media. Only callus proliferation was observed.

Calli developed from cotyledon in 'A' media showed shoot induction in two tubes after 15-17 days of culture 'E' medium. Root induction was also observed simultaneously in one tube but root did not developed further and browning of root started. Further sub-culture in root inducing media 'RL' produced roots and complete plantlets were obtained in 2 out of 8 cases. However, none of the plants could survive during the process of transfer.

Hypocotyl induced calli in 'A' media showed positive response towards organogenesis and shoots were found emerging 12-15 days after sub-culture. The shoots were multiplied by one more round of sub-culture in 'E' media. The shoots were splita nd sub-cultured in root induction media 'RL', where successful rooting was observed in 37.5% cases. *In-vitro* flowering was also observed in this case. These plantlets could be successfully transferred to the field and success rate was 25%.

The calli formed in 'A' medium from root showed positive response towards shoot induction. These shoots were also multiplied in E media and sub-cultured in 'RL' media for root induction. Success was obtained in development of plantlets and also transfer to field.

4.1.2. *T. subterraneum*

Seeds of *T. subterraneum* (genotype IG 96-112) were germinated on MS media devoid of any growth hormone under aseptic conditions. Most of the seeds (75%) developed into seedlings. The seedlings attained a height of 3-3.5 cm in 20-22 days. At this stage, explants like collar, hypocotyl, petiole, leaf, cotyledon and root were used for callus induction on different media and results are presented in Table 4.3.

Leaf : The low hormone medium 'A' failed to induce callus in most of the leaf explant. The enlargement of the explants was observed in only 11.6% cases where

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Table 4.3 : Callusing response of *T. subterraneum* explants in different medium

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Leaf	A	27	11.7+11.18	Compact	Yellow	Slow
	D	30	93.3+14.91	Compact	Yellow	Slow
Petiole	A	30	10.0+14.91	Friable, compact	Yellow	Slow, Medium
	D	25	72.0+30.33	Friable	Pale Green	Medium
Cotyledon	A	28	11.3+17.58	Compact	Yellow	Slow
	D	30	53.3+38.01	Compact	Yellow	Slow
Hypocotyl	A	30	6.7+9.13	Friable	Yellow	slow
	D	30	66.7+16.67	Friable	Green	Medium
Collar	A	30	50.0+20.41	Compact	Green	Slow
	D	30	66.7+11.79	Compact	Yellow	Medium
Root	A	30	50.0+16.67	Nodular	Yellow	Slow
	D	30	93.3+9.13	Nodular	Pale Green	Medium

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minute calli developed after 20-22 days of culture. The calli were compact in nature and yellow in colour.

More than 90% of leaf explants responded positively for callus induction in 'D' medium. The explants enlarged and thickened within 10-12 days of culture. Callus was initiated after 15 days of culture. Callus formation was observed initially at the cut ends of the midrib which later on spread along the periphery and in 33 days the callus covered the explant but did not proliferate much after that. The callus was compact, lacked pigment and was yellow in colour. The growth after 35 days was only 3-3.5 mm.

Petiole : In 'A' medium only few petiole explants (10%) showed swelling which further developed into calli. The calli were friable /compact in nature and slow growing. Calli remained yellow and failed to develop chlorophyll pigmentation.

Swelling in petiole explants cultured in 'D' medium was induced in about 6-7 days and small callus was formed in 12-13 days. Callus induction was first observed at the cut ends which later on proliferated and covered whole of the explant. Callus induction was recorded in 72% of explants. The callus was friable and pale green in colour. The growth rate recorded was high and the calli reached the size of about 6-6.5 mm diameter in about 32 days. At this stage the callus was sub cultured.

Cotyledon : In 'A' medium having low auxin and cytokinin the cotyledons showed callus induction in 11.3% explants. Calli were compact and yellow in nature and slow growing. Necrosis of the tissues in remaining cultures was observed after 12-15 days of inoculation.

No change was observed in first 7 days of culture of cotyledon explants in 'D' medium. The thickening and initiation of pale yellow callus was observed at the edges of the explant after 15-16 days. The callus was compact and reached the size of 3 -3.5 mm in 32 days. This shows that growth rate was quiet slow in this case. Fifty three percent cotyledon explants showed callusing.

Hypocotyl : In 'A' medium the hypocotyl explants showed swelling in few cases

only and most of the explants showed discoloration and browning of the tissues after 12-15 days of culture. Very few explants (6.67%) responded for callusing with slow growth rate.

In 'D' media 67% explants responded positively for callus induction and proliferation. Initially the callusing was observed at the cut ends of the explants which later on spread along the surface of the explants. In this media, two different response in nature and character of calli was observed. Some calli were hyaline, friable and very slow growing as the callus was initiated after 15 days and reached to the size of 2.5 mm in 37 days. Whereas, in other group they turned green after 20 days of inoculation and growth rate was medium.

Collar : In another set, the collar explants were placed on low hormone 'A' media. The calli were induced in 50% collar explants. Nature of the calli was compact as in 'D' media but they were green and slow growing. The calli attained 3 mm size in 35 days and were sub-culture.

Collar explants placed on 'D' media induced swelling in 7-8 days which turned into small calli in 12 days. Positive response for callus induction was observed in 67% cases. The calli were compact and yellow in colour. The callus growth in first 20 days was medium and it proliferated up to 3 mm diameter. Later on the growth rate slowed down and in next 15 days only slight callus proliferation was observed. At this stage the callus was subjected to sub-culture.

Root : The low hormone medium 'A' induced callusing in 50 percent root explants. Nature of the callus was same as in 'D' medium i.e. nodular but the colour was yellow and rate of growth was slow. The callus size recorded after 35 days of culture was 3.5-4 mm in diameter.

Response of root explants for callus induction was high in 'D' medium. More than 90% of root explants formed nodular, pale green calli of 4.5-5 mm diameter in 35 days. The growth rate was medium.

Organogenesis : The calli obtained from all the explants viz. hypocotyl, root, collar petiole, cotyledon and leaf were split and sub-cultured in 10 tubes each

containing shoot inducing media 'E'. The response was not good except in the calli obtained from hypocotyl (Table 4.4).

In calli obtained from hypocotyl in 'D' media, no response was observed in 30% of tubes whereas in another 30% tubes initial proliferation at slow rate was found. Later on these calli turned brown and no organogenesis was observed even after repeated sub-culturing. In 40% tubes calli responded positively for organogenesis and shoot emergence was observed after 12-17 days of sub-culturing. Multiple shoot emergence was induced by one more sub-culturing in same medium.

Multiple shoots were then split in 2-3 tubes and were sub-cultured in root inducing medium (RL). After 10-15 days root initiation was observed in 50% shoots. Root growth rate was high and after 30 days of sub-culturing in RL medium, the complete plantlets were found. These plantlets were then hardened and transferred to field as per the method described earlier. Success was achieved in transferring 33.3% of plantlets. However, 66.7% plantlets could not be transferred as they died during the process of hardening.

Embryogenesis : The calli obtained from all six explant sets from 'D' media were also inoculated for induction of somatic embryoids in SEIM medium). In general, response towards embryogenesis was very poor. In 66.7 to 92.9 % cases of calli developed from different explants showed no response. (Table 4.4). Only in few cases, callus proliferation was recorded. Embryoid like structures were observed in hypocotyl, callus, petiole and leaf explants. These embryoids however, could not be developed further as they turned brown.

4.1.3. *T. repens*

Healthy, mature seeds of *T. repens* (genotype EC 400986) were allowed to germinate on MS media without hormonal supplement. The seeds germinated within 8-12 days. The germination was about 55-60%. In 25-28 days the seedlings attained the height of 3-3.5 cm. At this stage, collar, hypocotyl, leaf, cotyledon and root were used as explants source for callus induction in different media and results are presented in table 4.5.

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Table 4.4 : Subculturing response in *T. subterraneum*

Explant source	Callusing media	Response of callus to shooting media (E) on subculture (%)				Response of callus to media (SEIM) on subculture (%)			Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		NR	CP	Shoot ing	Root ing	NR	CP	Embryo genesis	NR	Shoot ing	Root ing		
Leaf	A	80.0	20.0	-	-								
	D	100.0	-	-	-	73.3	20.0	6.7					
Petiole	A	100.0	-	-	-								
	D	10.0	70.0	-	20.0	80.0	13.3	6.7					
Hypocotyl	A	100.0	-	-	-								
	D	30.0	30.0	40.0	-	83.3	8.3	8.3	33.3	16.7	50.0	50.0	33.3
Cotyledon	A	100.0	-	-	-								
	D	20.0	80.0	-	-	92.9	7.1	-					
Collar	A	80.0	20.0	-	-								
	D	90.0	10.0	-	-	66.7	20.0	13.3					
Root	A	80.0	20.0	-	-								
	D	100.0	-	-	-	85.7	14.3	-					

NR = No response

CP = Callus proliferation

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Table 4.5 : Callusing response of *T. repens* explants in different medium

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Leaf	A	27	0	-	-	-
	D	30	80.0±21.73	Compact	Green	Slow
Petiole	A	30	100	Friable /nodular	Green	Slow/ Medium
	D	30	76.7±9.13	Friable	Yellow	Slow
Cotyledon	A	30	6.7±14.05	Compact	Yellow	Slow
	D	29	96.0±8.94	Compact	Green	Slow
Hypocotyl	A	27	3.3±7.45	Compact	Yellow	Slow
	D	30	6.7±14.91	Friable	Pale Green	Slow
Collar	A	24	0	-	-	-
	D	24	0	-	-	-
Root	A	30	0	-	-	-
	D	30	0	-	-	-

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Leaf- Low hormone level media 'A' did not show any callusing response in any of the leaf explants. Only thickening of the leaves was observed which later on turned brown. No further response was noticed in this media.

Response of leaf explants for callusing was high in 'D' medium wherein 80% explants responded positively for callus induction. Explants thickened in 9-10 days and small, compact and green callus was formed in about 18-20 days, which further did not increase much in size but gave out numerous hairy outgrowths after 24 days of culture. After this the growth of the callus was checked and it did not show any further growth. Hairy outgrowth also did not differentiate further and turned brown.

Petiole- The low hormone medium 'A' responded positively for callus induction in petiole explants. Chlorophyllous callus was friable/ nodular. The growth rate was slow to medium.

In high hormone medium 'D', 76.7% explants responded positively for callus induction. The swelling in the explants was observed in about 20 days and small, friable, yellow callus was formed in about 24-26 days. The callus remained yellow and very slow growing i.e. its size was 3-3.5 mm in diameter in 35-38 days of culture.

Cotyledon- Very poor response for callusing was observed in the cotyledonary leaf explants in 'A' media. However, in 'D' medium more than 90% explants were able to develop callus. Small, green, compact callus was formed in 18-20 days which gave hairy outgrowths after 24-28 days. The growth of the callus was checked after this and browning of the explants and hairy outgrowth started.

Hypocotyl- Hypocotyl explants responded poorly in both 'D' and 'A' media. Only initial swelling was observed in few explants placed on 'A' media which later on (after 8-9 days) turned brown. In 'D' medium, callus was induced in only 6.7% explants. The callus was friable and slow growing, devoid of any green pigmentation. Swelling was noticed only after 7-8 days and very minute callus size (1.5-2.0 mm) was achieved even after 5 weeks of culture. In 'A' medium, compact, slow growing, yellow callus was formed in only 3.3% explants. The

callus initiation started after 12-14 days of inoculation and reached the size of 2-2.5mm in diameter after 5 weeks of inoculation.

Collar- In both 'D' and 'A' media collar explant failed to show response for callus induction and browning of the tissue was observed.

Root- Root explants failed to show any positive response towards callusing in any medium. The cultured explants showed necrosis after 5-7 days of culture.

Organogenesis- The callus obtained from hypocotyl, petiole, leaf, cotyledon were split and sub-cultured in 10 tubes each containing 'E' medium. Response of callus to organogenesis was not good in explants derived from hypocotyl, cotyledon and leaf. In most cases, calli showed only slight proliferation in the callus size after 5-6 days of culture but along with the proliferation, discolouration of the callus was also observed which then turned brown in 9-11 days.

Callus developed from petiole in both 'A' and 'D' media showed good response and shoot emergence was observed after 8-10 days of culture in 'E' (Table 4.6). Multiple shooting was recorded in further 6-8 days. After 30 days of sub-culturing in 'E' media the shoots were transferred to root inducting medium 'RL' ('RL' basal +0.21 mg/l IAA). This medium induced roots in the shoots obtained from callus initially developed on 'D' media in about 10-11 days after sub-culture. The regenerants thus obtained after total of 13-14 weeks were allowed to develop and put to hardening and transferred to soil

4.1.4 . *T. hybridum*

Healthy seeds of *T. hybridum* (genotype EC 401702) were germinated on MS media devoid of any hormone. The germination of the seeds was 55% and the seedlings attained the height of about 3-3.5 cm in 20-24 days. At this stage the explants like collar, hypocotyl, petiole, leaf, cotyledon and root were excised from the seedlings and inoculated on the various media for induction of callus and results are presented in Table 4.7, plate 6.

Leaf : 'A' and 'D' media seemed to have same effect on callus induction in leaf explants as thickening of the explants was observed in 9-11 days and pale-green.

Table 4.6 : Subculturing response in *T. repens*

Explant source	Callusing media	Response of callus to shooting media (E) on subculture (%)				Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		NR	CP	Shooting	Rooting	NR	Shooting	Rooting		
Leaf	D	80.0	20.0	-	-					
Petiole	A	50.0	20.0	30.0	-	38.8	61.2	-		
	D	30.0	40.0	30.0	-	13.3	53.3	33.3	33.3	40.0
Hypocotyl	A	100.0	-	-	-					
	D	90.0	10.0	-	-					
Cotyledon	A	100.0	-	-	-					
	D	20.0	80.0	-	-					

NR = No response   CP = Callus proliferation

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Table 4.7 : Callusing response of *T. hybridum* explants in different medium

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Leaf	A	27	100	Compact	Pale Green	Slow
	D	25	100	Compact	Pale Green	Slow
Petiole	A	30	93.3±9.13	Friable	Yellow	Slow
	D	30	93.3±14.91	Friable	Green	Slow
Cotyledon	A	27	0	-	-	-
	D	30	50.0±11.79	Compact	Hyaline	Slow
Hypocotyl	A	25	4.0±8.94	Friable	Yellow	Slow
	D	27	92.7±10.11	Friable	Green	Medium
Collar	A	27	100	Compact	Yellow	Slow
	D	28	100	Compact	Pale Green	Slow
Root	A	27	81.3±1.83	Friable	Yellow	Slow
	D	30	50.0±11.79	Compact	Hyaline	Slow

compact callus was formed at the edges of the explants in 18-20 days. The callus formed was very slow growing and in both the media root like outgrowth were observed in 30 days old callus.

Petiole- In medium 'A' (low hormone) though the percentage of explant response was high, the callus growth rate was slow as it reached a size of 3-3.5 mm in diameter after 35 days. Callus was friable and yellow and failed to develop green colour.

In 'D' medium, almost all the explants responded for callus induction, the swelling in the explants was initiated in 7-9 days and small, green, friable, callus was formed in 11-13 days. Further, the growth rate was slow as in 35 days the size of the callus was 4-4.5 mm in diameter.

Cotyledon : No response of cotyledon explants was observed in 'A' media. However, good response (50%) was observed in 'D' media. The swelling of explants was recorded after 6-7 days and compact, slow growing calli were developed. The calli remained hyaline.

Hypocotyl- The hypocotyl explants did not respond positively for callus induction in low hormone medium 'A'. Swelling was induced in a few explants in 8-9 days which failed to develop into callus even after 12-13 days of inoculation. Later on, necrosis of the tissue was observed.

In medium 'D' having high level of auxin and cytokinin, swelling initiated in 5-6 days and small green, friable callus was formed in 11-13 days in 93% explants. Further, proliferation of callus up to 12-13 mm in diameter was observed.

Collar- All the explants responded positively for callus induction in both 'D' and 'A' media. In low hormone medium 'A' callus was induced in 11-14 days. The callus formed was compact, pale yellow and very slow growing but browning of the calli started in 18-22 days in all the explants.

In high hormone level medium 'D', swelling was induced in 5-6 days and later on small, pale green callus was formed in 9-11 days, but after that the callus

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growth was checked and except a few hyaline out growths. no other change in the callus took place. After 13-14 days of culture, browning of the calli started.

Root- The callusing response in root explants in low auxin and cytokinin media ('A' media) was 81% and the swelling in the explants was observed after 12-15 days and small, pale yellow, friable callus was formed in 25-28 days, no further growth of the callus was observed in next 10 days and browning of the callus started.

In 'D' medium, about 50% explants responded for callus induction. Small, hyaline callus was formed in about 28-30 days, emergence of hyaline, hairy out growths was also observed at this stage. Further growth of the callus was checked and browning started.

Organogenesis- The calli obtained from all the six explant source were subjected to sub-culture in shooting medium 'E'. Callus obtained from explant sources like root, collar, cotyledon and leaf did not show any shoot growth. Root like white outgrowths were recorded in some cases in calli developed from cotyledon and petiole in 'D' media.

However, the callus obtained from hypocotyl responded positively after sub-culturing in shoot inducing medium.. The hypocotyl derived callus developed on 'D' and 'A' medium when sub-cultured after splitting showed proliferation of the callus in first 10-15 days. Shoots were initiated in the calli obtained from both 'D' and 'A' media (Table 4.8). Response of calli initially induced on 'D' media was better (40%) for shooting as compared to that of 'A' media (20%). The shoots were multiplied and then transferred to root inducing media ('RL'). Very good response of rooting of these shoots was observed and more than 70% shoots developed roots. These complete plantlets were hardened and transferred to soil. Success rate of transferring to field was similar in both cases.

Embryogenesis- Calli obtained from all six explants were also sub-cultured on 'SEIM' for induction of embryoids. In hypocotyl and cotyledon derived calli no positive response was observed. Poor response was noticed in root. collar and leaf derived calli (Table 4.8). Petiole derived calli showed better response for embryoid

Table 4.8 : Subculturing response in *T. hybridum*

Explant source	Callusing media	Response of callus to shooting media (E) on subculture (%)				Response of callus to media (SEIM) on subculture (%)			Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		NR	CP	Shooting	Rooting	NR	CP	Embryogenesis	NR	Shooting	Rooting		
Leaf	A	100.0	-	-	-	38.5	53.8	7.7					
	D	10.0	90.0	-	-	58.3	33.3	8.3					
Petiole	A	60.0	-	40.0	-	61.5	7.6	30.7	80.0	20.0	-		
	D	30.0	60.0	-	10.0	63.6	9.1	27.3					
Hypocotyl	A	10.0	70.0	20.0	-				26.7	-	73.3	73.3	36.7
	D	10.0	50.0	40.0	-	63.5	37.5	-	13.3	-	86.7	86.7	38.5
Cotyledon	D	70.0	10.0	-	20.0	55.6	44.4	-					
Collar	A	100.0	-	-	-	77.8	22.2	-					
	D	100.0	-	-	-	40.0	53.3	6.7					
Root	A	90.0	10.0	-	-	30.0	60.0	10.0					
	D	90.0	10.0	-	-	50.0	50.0	-					

NR = No response   CP = Callus proliferation

induction. However, these embryoids when sub-cultured, showed browning of the tissue and could not be maintained.

4.1.5. *T. apertum*

Seeds of genotype (EC 401712) on MS medium without any hormone supplement germinated in 9-10 days and germination were 65%. The germinated seedlings attained a height of 3-3.5 cm in 20-24 days. At this stage, explants like collar, hypocotyl, petiole, root, cotyledon and leaf were taken for callus induction on different media and results are presented in Table 4.9, plate 5.

Leaf- Leaf explants did not respond for callus induction in 'A' medium. Only slight swelling was induced in about 7-8 days which was followed by browning at the edges. The browning later on spread all over the explant. In 'D' medium, 14% explants responded positively for callus induction. Slow growing compact calli were formed which turned pale green in colour 20-25 days after inoculation.

Petiole- Low hormone medium 'A' failed to induce callus in petiole explants and tissue browning was observed in all the explants in 12-14 days.

The explants cultured in 'D' medium responded positively for callus induction and proliferation. Swelling initiated in 5-7 days of culture and small, green and friable callus was formed in about 14-16 days which later on proliferated and reached the size of 6-6.5 mm diameter in 35 days. At this stage, a few hyaline outgrowths emerged from the callus.

In L' medium only 33.3% explants showed positive response towards callus induction. The callus was slow growing, friable and pale green in colour. The callus grew to 3-3.5 mm in diameter in 17-18 days.

In 'P' medium the response for callus induction from petiole explants was good (27.3%). Small, pale yellow, friable callus was formed in about 16-18 days which later on slightly increased in size but no change in colour took place. The callus was sub-cultured at this stage.

Cotyledon- The explants failed to respond in low hormone medium 'A'. Few

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Table 4.9 : Callusing response of *T. apertum* explants in different medium

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Leaf	A	24	0	-	-	-
	D	23	14.0±21.91	Compact	Pale Green	Slow
Petiole	A	24	0	-	-	-
	D	28	96.7±7.45	Friable	Green	Medium
	L	30	13.0±12.04	Friable	Yellow, Pale Green	Slow
	P	27	21.0±14.32	Friable	Yellow	Slow
Cotyledon	A	30	0	-	-	
	D	28	53.3±10.27	Compact	Yellow	Slow
Hypocotyl	A	30	6.7±14.91	Friable	Pale yellow	Slow
	D	30	93.3±14.91	Friable	Green	Medium
	L	27	47.3±20.60	Friable	Pale Green	Slow
	P	27	9.0±12.45	Friable	Yellow	Slow
Collar	A	30	0	-	-	-
	D	30	0	-	-	-
Root	A	30	0	-	-	-
	D	30	10.0±14.91	Compact	Pale Green	Slow

explants showed slight swelling which later on turned brown. But in high levels of auxin and cytokinin medium 'D', 53.3% explants were able to respond positively for callus induction and proliferation. The explants thickened in 8-10 days and very small, pale yellowish, compact calli were formed at the edges of the explants. Within 28-30 days the callus covered whole of the explant, but the callus did not increase much in diameter.

Hypocotyl- Explants of about 3-3.5 mm length were excised and inoculated in the two media. In low hormone medium 'A', the explants showed only initial swelling after 16-18 days. Very few explants showed callus induction. Necrosis of the tissue was observed after 12-15 days of inoculation in most of the cases.

In high hormone media 'D', swelling was induced in about 6-8 days and small callus was initiated in 12-14 days. Callus was first formed at the cut ends of the explants which later on spread along the surface of the explants. After 32 days, friable bright green callus of 6-6.5 mm diameter was formed showing medium rate of growth. More than 90% explants responded for callus induction and proliferation.

In the medium 'L' the auxin NAA was replaced by picloram (L_2 basal+0.06 mg/l picloram +0.1 mg/l BAP). In all, 47.3% of the explants responded for callus induction. Swelling was induced in about 10-12 days and small pale green, friable callus was formed in about 20-22 days.

In the medium 'P' the quantity of picloram and BAP was increased (L_2 basal+0.6 mg/l pic. +1.0 mg/l BAP). In this medium response was quite low and only 6.7% of the hypocotyl explants responded for callus induction. Theyellow and friable calli formed after 25-28 days were of slow grwoth rate and attained a size of 3-3.5 mm in diameter..

Collar- Both high hormone medium 'D' and low hormone medium 'A' failed to induce callus from collar explants. Slight swelling was induced in few explants in 16-18 days which failed to develop into callus even after 28-30 days and after that tissue turned brown.

Root : Response of root explants was very poor. Only in 'D' media 10% explants showed callus induction. The callus was compact, pale green and slow growing.

Organogenesis- The callus obtained from the explants like root, hypocotyl, cotyledon, petiole, leaf were split and sub-cultured in 10-15 tubes containing shoot inducing media 'E'. The response of 'E' media was not good except in the calli obtained from petiole which was initially induced on 'D' media. Shooting was recorded in 50% tubes in this case (Table 4.10). Shoots were multiplied by one more round of sub-culturing after 30 days in 'E' media. The shoots were split and sub-cultured in root inducing 'RL' media. Success was achieved in most of the cases (86.7%),where complete plantlets with root and shoot could be obtained. The plantlets were hardened and transferred to field. The percentage of success being 26.7%.

Embryogenesis- The calli obtained from hypocotyl and petiole from 'D' media were subjected to sub-culturing in embryogenic media 'SEIM' also. In hypocotyl derived calli, except for one tube only callus proliferation was observed (Table 4.10). Whereas, calli from petiole showed better response and embryoids could be induced in this combination. Callus from petiole initially induced in 'D' media proliferated in first 10-12 days and few hyaline outgrowths were also visible on the surface. These hyaline outgrowths turned bright green after 15-18 days.

These callus showing embryoid like outgrowths were split in 5 tubes each of 'E' and 'SEIM' medium. Initiation of shoots was observed after 12-15 days, which when sub-cultured in 'RL' medium showed root emergence in about 6-8 days. Therefore, full regenerants were obtained which were hardened and transferred to soil

4.1.6. *T. glomeratum*

Seeds of *T. glomeratum* (genotype EC 401700) were germinated on MS medium without any hormone supplement under aseptic condition. The seeds germinated within 9-10 days of culture. Germination was 70% and the seedlings attained the height of 3-3.5 cm in 20-22 days. Explants like hypocotyl, petiole, leaf, collar, cotyledon and root were excised from the seedlings and were cultured

Table 4.10 : Subculturing response in *T. apertum*

Explant source	Callusing media	Response of callus to shooting media (E) on subculture (%)				Response of callus to media (SEIM) on subculture (%)			Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		NR	CP	Shooting	Rooting	NR	CP	Embryo genesis	NR	Shooting	Rooting		
Leaf	D	100.0	-	-	-								
Petiole	D	30.0	20.0	50.0	-	41.7	16.7	41.7	13.3	-	86.7	86.7	26.7
	L	60.0	40.0	-	-								
	P	50.0	40.0	-	-								
Cotyledon	D	75.0	25.0	-	-								
Hypocotyl	A	100.0	-	-	-								
	D	10.0	90.0	-	-	83.3	8.3	8.3					
	L	70.0	30.0	-	-								
	P	90.0	10.0	-	-								
Root	D	81.2	18.2	-	-								

NR = No response   CP = Callus proliferation

on different media combinations for callus induction and results are presented in Table 4.11, Plate 4.

Leaf- Leaflets of young seedlings were used as explant source and small pieces were cultured on 'A' as well as 'D' media. In both the cases very poor response of the leaf explants in inducing callus was observed. In most of the cases necrosis of explants was observed within 8-10 days and the explants were discarded.

Petiole - In 'D' medium, 73.3% explants induced swelling in 10-11 days and small, green, friable callus was formed in about 18-20 days. Callus size after 33 days was observed to be 6-6.5 mm in diameter. Very poor response for callusing was observed in petiole explants in 'A' medium containing low auxin and BAP even after 25-30 days of culture and all the explants showed necrosis of the tissue.

Cotyledons- Cotyledon explants responded positively for callusing in 'D' medium. The explants enlarged and thickened in 7-8 days and very small callus, yellow in colour was formed in 12 days on the edges of the explant which after 16-18 days covered the whole explant. The callus thus formed was not able to develop green colour even after 25-27 days and remained yellow in colour, compact and slow growing.

In 'A' medium, most of the explants did not develop into callus and only slight swelling of the tissue was observed in few cases (3.3%). Most of the explants showed browning of the tissues within 10 days.

Hypocotyl - About 3 to 4 mm long pieces of hypocotyl were taken for callus induction. High concentration of hormones in 'D' medium seems to have negative effect on callus induction in hypocotyl explants. Explant showed slight swelling after 15-18 days of culture which did not develop further and all the other explants showed necrosis of the tissue. However, in 'A' medium containing quite low concentrations of growth hormones, explants responded for callus induction. Swelling was induced in about 10-11 days and small compact, yellow callus was formed in 20-24 days. The callus was slow growing and developed into callus mass of 4.5 -5 mm diameter in 5 weeks.

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Table 4.11 : Callusing response of *T. glomeratum* explants in different medium

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Leaf	A	30	6.7+9.13	Compact	Yellow	Slow
	D	30	3.3+7.45	Compact	Yellow	Slow
Petiole	A	27	4.0+8.94	Friable	Yellow	Slow
	D	27	73.3+19.00	Friable	Green	Medium
Cotyledon	A	30	3.3+7.45	Friable	Hyaline	Slow
	D	30	93.3+14.91	Compact, nodular	Yellow	Slow
Hypocotyl	A	30	66.7+11.79	Compact	Yellow	Slow
	D	30	3.3+7.45	Friable	Hyaline	Slow
Collar	A	25	0	-	-	-
	D	26	0	-	-	-
Root	A	27	0	-	-	-
	D	30	0	-	-	-

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Collar – Collar explants did not show any response for callus induction in both the media combinations. Browning of explants was observed within 7-10 days of inoculation.

Root- Root explants did not show any callus induction in either 'D' or 'A' media. Tissue browning was observed after 7-8 days hence the explants were discarded.

Organogenesis : The callus obtained from hypocotyl, petiole, cotyledon and leaf explants were split and sub-cultured in 10 tubes containing 'E' media.

The response of hypocotyl, leaf and cotyledon derived callus was not good. Only 20%, 10% and 30% calli respectively showed slight callus proliferation and no sign of organogenesis was observed even after repeated sub-culturing (Table 4.12). Browning started along the edges of the callus within 11-12 days and no further growth was observed.

The callus developed from petiole on 'D' media showed emergence of small shoot like structure within 15 days of sub-culturing in 'E' media. The multiple shoot formation was observed in these tubes in next 10-12 days. These multiplied shoots were then split in many tubes and sub cultured on root inducing media 'RL' ('RL' basal +0.21 mg/l IAA) after total of 35-40 days of sub-culturing in 'E' media. Root initiation was first noticed within 7-9 days followed with emergence of multiple roots and secondary roots in next 10 days. Thus complete plantlets (with root and shoot) were obtained in about 14 weeks. These plantlets were then hardened and transferred to field.

Embryogenesis - The calli of three explants hypocotyl, petiole and cotyledon were sub-cultured in SEIM media but the calli from cotyledon and petiole did not show any sign of embryoid formation. Only callus proliferation was seen which too turned brown after 20-25 days. In the calli obtained from hypocotyl on 'A' media some embryoid like structure was recorded in one tube (Table 4.12). However, this could not be further used as they turned brown after 20 days.

Table 4.12 : Subculturing response in *T. glomeratum*

Explant source	Callus ing media		Response of callus to shooting media (E) on subculture (%)				Response of callus to media (SEIM) on subculture (%)			Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
			NR	CP	Shoot ing	Root ing	NR	CP	Embryo genesis	NR	Shoot ing	Root ing		
Leaf	A		90.0	10.0	-	-								
	D		100.0	-	-	-								
Petiole	A		100.0	-	-	-								
	D		10.0	40.0	50.0	-	40.0	60.0	-	20.0	20.0	60.0	60.0	33.3
Hypocotyl	A		80.0	20.0	-	-	50.0	40.0	10.0					
	D		100.0	-	-	-								
Cotyledon	A		100.0	-	-	-								
	D		70.0	30.0	-	-	42.9	57.1	-					

NR = No response   CP = Callus proliferation

4.2. *In vitro* response in different genotypes of *T. alexandrinum*

4.2.1. *T. alexandrinum* genotype FAO-1 (JHB 97-1)

Seeds of *T. alexandrinum* genotype FAO-1 were grown on MS media without any hormonal supplementation. Germination was 92%. The explants were excised at two growth stages of seedlings *i.e.* Young (10 days old) and Old (30 days old) and placed on four media preparations 'A', 'B', 'C' and 'D' and results are given in Table 4.13 to 4.16, plate 7.

Leaf - Leaf explants did not respond to 'A'; media at all whereas it showed little response to 'D' media irrespective of age of explants. However, the explants on 'B' and 'C' media showed positive response and 23 to 70% explants developed callus. Mostly the calli were friable in nature except a few calli which were nodular in nature. All the calli were green in colour, however, growth varied from slow to medium.

Petiole - The over all response of petiole explants from young seedling was better as compared to old seedling explants. 13 to 80% explants developed calli in young explants. Among the four media, 'B' and 'C' were more effective and 56 to 80% explants excised from old as well as young seedlings responded for callusing. Most of the calli developed were friable in nature except those growing on 'D' media which were compact in nature. Mostly the callusing from petiole was slow and green in colour.

Hypocotyl - Hypocotyl explants from old as well as young seedlings showed positive response for callusing on two media combinations *i.e.* 'B', and 'D'.

In 'A' media young explants showed 90% response for callus induction whereas old explants responded poorly (13%).

In general, the response of young explants was better as compared to old seedling explants in three media ('A', 'B' and 'D') whereas, in 'C' media old explants showed better response (66.7%) as compared to young explants (10%).

Invariably all the calli were friable in nature. Growth of callus developed from young explants was high on 'C' and 'D' media where as in all other cultures

Table 4.13: Callusing response of *T. alexandrinum* explants in different medium

Genotype – FAO-1   Age of explant – Young seedling

Explant Source	Media	No. of explants cultured	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	30.0 ± 13.94	Friable	Yellow, Green	Slow
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	16.7 ± 11.79	Compact	Green	Medium
Hypocotyl	A	30	90.0 ± 9.13	Friable	Yellow, Green	Slow, Medium
	B	30	93.3 ± 9.13	Friable	Yellow, Green	Slow, Medium
	C	30	10.0 ± 9.13	Friable	Green	High
	D	30	70.0 ± 7.45	Friable	Green	High
Petiole	A	30	13.3 ± 13.94	Friable	Hyaline	Slow
	B	30	63.3 ± 7.45	Friable	Green	Slow
	C	30	80.0 ± 7.45	Friable	Yellow	Medium
	D	30	23.3 ± 9.13	Compact	Green	Slow
Leaf	A	30	0	-	-	-
	B	30	66.7 ± 11.79	Friable	Green	Slow
	C	30	70.0 ± 7.45	Friable, Nodular	Yellow, Green	Slow, Medium
	D	30	23.3 ± 9.13	Friable	Green	Slow

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	88022.259	5868.1506	71.9308**
A. Media	3	6760.6542	2253.5514	27.6237**
B. Explant	3	29871.07	9957.0232	122.0515**
AxB	9	51390.535	5710.0594	69.9929**
Within sub group (error)	64	5221.1515	81.5805	

** = Significant at 1% level.

Table 4.14 : Subculturing response in *T. alexandrinum*

Genotype – FAO-1   Age of explant – Young seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					
		Media	NR	CP	Shooting	Rooting	
Collar	A	E	100.0	-	-		-
	D	H	92.3	7.7	-		-
Hypocotyl	A	E	30.0	70.0*	-		-
	B	F	100.0	-	-		-
	C	G	100.0	-	-		-
	D	H	45.5	54.5	-		-
Petiole	A	E	100.0	-	-		-
	B	F	100.0	-	-		-
	C	G	69.2	30.8	-		-
	D	H	7.7	92.3	-		-
Leaf	B	F	93.3	6.7	-		-
	C	G	86.7	13.3	-		-
	D	H	42.9	57.1	-		-

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

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Table 4.15: Callusing response of *T. alexandrinum* explants in different medium

Genotype - FAO-1   Age of explant – Old seedling

Explant Source	Media	No. of explants cultured	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	66.7±11.79	Compact, Nodular	Yellow, Green	Slow, Medium
	B	30	66.7±20.41	Friable, Nodular	Yellow, Hyaline	Medium
	C	30	50.0±11.79	Friable, compact	Green	Medium
	D	30	100	Compact	Green	High
Hypocotyl	A	30	13.3±13.94	Friable	Green	Medium
	B	30	36.7±13.94	Friable	Yellow, Green	Slow, Medium
	C	30	66.7±20.41	Friable	Green	Slow
	D	30	63.3± 7.45	Friable	Green	Slow
Petiole	A	30	16.7±16.67	Friable	Green	Slow
	B	30	63.3±13.94	Friable	Hyaline, Green	Slow, Medium
	C	30	56.7±25.28	Friable	Green	Slow
	D	30	0	-	-	-
Leaf	A	30	0	-	-	-
	B	30	26.7±25.28	Friable	Green	Slow/Medium
	C	30	40.0±9.13	Friable	Yellow, Green	Slow/Medium
	D	30	0	-	-	-

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	67000.28	4466.69	21.09**
A. Media	3	9750.00	3250.00	15.33**
B. Explant	3	30861.05	10287.02	48.57**
AxB	9	26389.22	2932.14	13.84**
Within sub group (error)	64	13555.62	211.81	

** = Significant at 1% level.

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Table 4.16 : Subculturing response in *T. alexandrinum*

Genotype – FAO -1   Age of explant –Old seedling

Explant source	Callusing media	Response of callus to sub-culture (%)			Response of callus to shooting media on (%)		Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting		
Collar	A	E	100.0	-	-	-					
	B	F	100.0	-	-	-					
	C	G	100.0	-	-	-					
	D	H	100.0	-	-	-					
Hypocotyl	A	E	30.0	70.0	-	-					
	B	F	10.0	90.0*	-	-					
	C	G	10.0	90.0*	-	-					
	D	H	10.0	90.0	-	-					
Petiole	A	E	10.0	30.0	60.0	-	20.0		80.0	80.0	58.3
	B	F	-	100.0	-	-					
	C	G	-	100.0	-	-					
Leaf	B	F	90.0	10.0	-	-					
	C	G	90.0	10.0	-	-					

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

slow to medium rate of growth was observed. The slow and medium growing calli were yellow to pale green in colour and the calli growing with faster rate were green in colour.

Collar - The collar explants excised from old seedlings showed better response for callusing and more than 50% explants developed in callus in all the four media as against 0 to 30% response of young explants. Calli were of different nature in different media combination. They were observed to be compact, friable and nodular in different sets. The growth of callus was slow to medium on 'A' media and callus thus formed was yellow to green in colour. Calli developing on 'D' media were green in colour, whereas calli from old explants developed on 'B' and 'C' media were yellow or hyaline.

Organogenesis - Calli obtained from young explants –media combination did not show any tissue differentiation on sub-culturing to shoot inducing media. Only callus proliferation was noticed. Embryoid like structure were observed in some tubes in hypocotyl induced callus on sub-culturing in 'E' media (table 4.14).

Response of calli induced from old explants also was not good. Embryoid like structures were seen on hypocotyl induced calli on sub-culturing to 'F' and 'G' media. Tissue differentiation and shoot formation was observed in petiole induced calli on sub-culturing to 'E' media. These shoots also showed good root induction in 'RL' media. Most of the shoots developed roots within 7 to 10 days in 'RL' media and the complete regenerants were then transferred to field after hardening. Successful transfer to field was obtained in plantlets derived from hypocotyl explants (Table 4.16).

4.2.2. *T. alexandrinum* genotype JHB 146

Seeds of *T. alexandrinum* (genotype JHB 146) were germinated on MS media without any hormonal supplementation and explants from collar, hypocotyl, petiole and leaves were excised at two growth stages *i.e.* young (10 days old) and old (30 days old seedling). Explants were inoculated on four callus inducing media 'A', 'B', 'C' and 'D' and response are given in table 4.17 and 4.19, plate 7, 8.

Table 4.17: Callusing response of *T. alexandrinum* **explants in different medium**

Genotype - JHB 146   Age of explant – Young seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	63.3±13.94	Friable	Green, Yellow	Slow, Medium
	B	30	16.7±11.79	Friable	Pale Green	Medium
	C	30	20.0±7.45	Friable	Yellow	Slow
	D	30	13.3±7.45	Friable	Yellow, Green	Slow
Hypocotyl	A	30	26.7±14.91	Friable	Green	Medium
	B	30	40.0±9.13	Compact	Green	Slow
	C	30	50.0±0.00	Friable	Yellow	Medium
	D	30	46.7±13.94	Friable	Yellow	Slow
Petiole	A	30	40.0±14.91	Friable	Green	Slow
	B	30	36.7±18.26	Friable	Green	Medium
	C	30	20.0±18.26	Friable	Yellow	Medium
	D	30	40.0±9.13	Friable	Green	Medium
Leaf	A	30	43.3±9.13	Nodular	Green	Slow
	B	30	23.3±9.13	Compact	Green	Slow
	C	30	26.7±9.13	Compact	Green	Medium
	D	30	20.0±7.45	Compact	Green	Slow

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	14984.41	998.960	7.193**
A. Media	3	2902.27	967.424	6.966**
B. Explant	3	2124.23	708.077	5.098**
AxB	9	9957.9	1106.43	7.967**
Within sub group (error)	64	8888.38	138.88	

** = Significant at 1% level

Table 4.18 : Subculturing response in _T. alexandrinum_

Genotype – JHB 146   Age of explant – Young seedling

Explant source	Callusing media	Media	Response of callus to shooting media on sub-culture (%)				Response of shoots to rooting media (RL) on subculture (%)		
			NR	CP	Shooting	Rooting	NR	Shooting	Rooting
Collar	A	E	50.0	50.0	-	-			
	B	F	20.0	80.0	-	-			
	C	G	20.0	80.0*	-	-			
	D	H	100.0	-	-	-			
Hypocotyl	A	E	10.0	90.0	-	-			
	B	F	100.0	-	-	-			
	C	G	20.0	60.0	20.0	-	50.0	50.0	-
	D	H	100.0	-	-	-			
Petiole	A	E	100.0	-	-	-			
	B	F	20.0	30.0	50.0	-	40.0	60.0	-
	C	G	100.0	-	-	-			
	D	H	30.0	70.0	-	-			
Leaf	A	E	10.0	90.0	-	-			
	B	F	100.0	-	-	-			
	C	G	10.0	90.0	-	-			
	D	H	100.0	-	-	-			

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

Table 4.19 :Callusing response of *T. alexandrinum* explants in different medium

Genotype - JHB 146   Age of explant – Old seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	96.7+7.45	Compact	Green	Medium
	B	30	26.7+9.13	Compact	Green	Medium
	C	30	23.3+14.91	Compact	Yellow	Slow
	D	30	0	-	-	-
Hypocotyl	A	30	50.0+20.41	Friable	Green	Slow, Medium
	B	30	86.7+21.73	Friable	Green	Medium
	C	30	43.3+9.13	Friable	Yellow	Slow
	D	30	40.0+9.13	Friable	Yellow	Slow
Petiole	A	30	0	-	-	-
	B	30	30.0+7.45	Friable	Pale Green	Slow
	C	30	23.3+14.91	Friable	Green	Slow
	D	30	0	-	-	-
Leaf	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	72688.95	4845.93	46.5242**
A. Media	3	9704.514	3234.84	31.0566**
B. Explant	3	35480.69	11826.9	113.5461**
AxB	9	27503.75	3055.97	29.3394**
Within sub group (error)	64	6666.2	104.1594	

** = Significant at 1% level

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Table 4.20 : Subculturing response in *T. alexandrinum*

Genotype – JHB 146   Age of explant – Old seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting		
Collar	A	E	53.8	46.2	-	-					
	B	F	64.3	35.7	-	-					
	C	G	80.0	20.0	-	-					
Hypocotyl	A	E	46.7	53.3	-	-					
	B	F	12.5	56.3	31.3	-	7.7	7.7	86.7	86.7	61.5
	C	G	6.3	75.0*	18.7	-	80.0	20.0	-		
	D	H	38.5	61.5	-	-					
Petiole	B	F	54.5	45.5	-	-					
	C	G	72.7	27.3	-	-					

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

Leaf - Response of young leaf explants varied from 20 to 43.3% in four different media. However, the explants taken from old seedlings did not respond to any of four media. Growth of callus was slow to medium. Callus growing on 'B', 'C' and 'D' media were compact in nature as compared to nodular callus on 'A' media.

Petiole - Young explants responded to all the four media and 20 to 40% explants developed into callus. But, the explants obtained from old seedling did not respond to 'A' and 'D' media. However, 23 to 30% explants developed callus on 'B' and 'C' media. Development of callus from petiole explants took about 25-30 days. Growth of callus from young explants on 'B', 'C' and 'D' media was medium as compared to slow growth of callus on 'A' media. In old explants induced callus growth rate was slow in both 'B' and 'C' media. In most of cultures callus formed was friable and green in colour.

Hypocotyl - The hypocotyl explants collected and inoculated at both growth stages responded well in all the four media and 26.7 to 86.7% explants developed into callus. Response of old explants was better in 'A' and 'B' media as compared to young explants. In first 12-15 days of inoculation only swelling of explants was noticed while in next 10-15 days it developed into bright green calli. Majority of calli formed were friable in nature except a few growing on 'B' media which were compact in nature. Mostly the callus development on 'A' and 'B' media was medium and developed green colour whereas on 'C' and 'D' media growth was slow and remained pale yellow.

Collar - The collar explants of both growth stages responded to 'A', 'B' and 'C' media and 16.7 to 96.7% explants developed into callus. Response of explants on 'D' media was poor in young explants (13.3%) whereas no response was observed in case of old explants. In most of cases, the growth was very slow and for initial 10-12 days of cultures, only swelling was observed. Later on, the explants showed slow growth of callus at cut ends and after 25 to 30 days of inoculation bright green callus was formed. Calli obtained from young explants were friable in nature whereas those from old explants were compact in nature. Mostly the callus developing on 'A' and 'B' media were green in colour whereas that on 'C' and 'D' media were pale yellow. In general, the slow growing callus did not develop green

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colour and callus developing at medium rate developed green colour.

Organogenesis - Young seedling collar and leaf explants induced callus resulted in only callus proliferation and no shoot initiation could be observed. In collar induced calli obtained on 'C' media, green globular embryoid like structure were noticed 25 days after subculture in 'G' media. However, on further sub culture these embryoids showed browning and necrosis.

Shoot formation was induced in hypocotyl derived calli on 'C' media and petiole derived calli on 'B' media when sub cultured on 'G' and 'F' media respectively. Shoot was formed in more than 50% tubes in both these cases (Table 4.18).

In old hypocotyl explant induced calli on 'B' and 'C' media successful shoot formation was induced on sub-culturing in 'F' and 'G' media respectively. Successful rooting and complete plantlet development was observed when these shoots were split and sub-cultured in 'RL' media. Root initiation started 15-20 days after sub-culturing in 'RL' media. Successful establishment rate in soil was also higher in shoots induced on 'F' media (Table 4.20, plate 11). In other combinations only callus proliferation was observed. In most of the cases calli turned brown 15-20 days after sub-culture.

4.2.3. *T. alexandrinum* genotype BL 142

Seeds of *T. alexandrinum* genotype BL 142 were grown on MS media without any hormonal supplementation and explants like collar, hypocotyl, petiole and leaves at two developmental stages *i.e.* young (10 days old seedlings) and old (30 days old seedlings) were cultured on four media and results of callus induction is given in tables 4.21 and 4.23, plate 7.

Leaf - The overall response of young leaf explants for callus on any four media was low and only 10 to 36.7% explants developed into callus. Growth of callus was slow to medium. In old explants callus induction was 33.3% in 'A' media whereas 'B', 'C', 'D' media did not show any positive response. Mostly callus was of compact nature and callus on 'A' and 'C' media developed green

Table 4.21: Callusing response of *T. alexandrinum* explants in different medium

Genotype - BL 142   Age of explant – Young seedling

Explant Source	Media	No. of explants cultured	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	23.3+9.13	Friable	Pale Green	Slow
	B	30	60.0+9.13	Friable	Yellow	Slow
	C	30	26.7+19.00	Friable	Yellow	Medium
	D	30	0	-	-	-
Hypocotyl	A	30	70.0+24.72	Friable	Green	Slow
	B	30	60.0+30.28	Friable	Yellow	Medium
	C	30	70.0+13.94	Friable	Green	Medium
	D	30	66.7+20.41	Friable	Pale Green	Medium
Petiole	A	30	50.0+0.00	Friable	Green	Slow
	B	30	23.3+17.00	Friable	Yellow	Slow
	C	30	56.7+9.13	Friable	Yellow	Medium
	D	30	30.0+27.39	Friable	Green	Medium
Leaf	A	30	10.0+9.13	Compact	Green	Slow
	B	30	20.0+18.26	Friable	Yellow	Slow
	C	30	30.0+21.73	Compact	Green	Medium
	D	30	36.7+13.94	Compact	Yellow	Slow

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	36690.89	2446.059	7.575**
A. Media	3	1426.875	475.625	1.473
B. Explant	3	21454.4	7151.466	22.147**
AxB	9	13809.61	1534.402	4.752**
Within sub group (error)	64	20666.4	322.9125	

** = Significant at 1% level

Table 4.22 : Subculturing response in *T. alexandrinum*

Genotype – BL 142   Age of explant – Young seedling

| Explant source | Callusing media | Response of callus to shooting media on sub-culture (%) | | | | |
		Media	NR	CP	Shooting	Rooting
Collar	A	E	83.3	16.7	-	-
	B	F	46.2	53.8	-	-
	C	G	23.1	76.9*	-	-
Hypocotyl	A	E	80.0	20.0	-	-
	B	F	26.7	73.7*	-	-
	C	G	36.4	63.6	-	-
	D	H	42.9	57.1	-	-
Petiole	A	E	72.7	27.3	-	-
	B	F	81.8	18.2	-	-
	C	G	18.7	81.3*	-	-
	D	H	6.67	93.3*	-	-
Leaf	A	E	90.9	9.1	-	-
	B	F	78.6	21.4	-	-
	C	G	28.6	71.4	-	-
	D	H	20.0	80.0	-	-

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

Table 4.23: Callusing response of *T. alexandrinum* explants in different medium

Genotype - BL 142   Age of explant – Old seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-
Hypocotyl	A	30	66.67±11.79	Friable	Pale Green	Slow
	B	30	50.00±0.00	Friable	Pale Green	Medium
	C	30	13.33±7.45	Compact	Yellow	Medium
	D	30	16.67±11.79	Friable. Compact	Yellow, Hyaline	Medium
Petiole	A	30	53.33±7.45	Friable	Yellow	Slow
	B	30	100	Friable	Green	Medium
	C	30	100	Friable	Green	Medium
	D	30	0	-	-	-
Leaf	A	30	33.33±16.67	Compact	Green	Slow
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	98096.99	6539.799	156.9709**
A. Media	3	15236.00	5078.668	121.9002**
B. Explant	3	49819.96	16606.65	398.5995**
AxB	9	33041.03	3671.225	88.1182**
Within sub group (error)	64	2666.40	41.6625	

** = Significant at 1% level

Table 4.24 : Subculturing response in *T. alexandrinum*

Genotype –BL 142   Age of explant –   Old seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting		
Hypocotyl	A	E	7.1	35.7	57.1	-	40.0	26.7	33.3	33.3	80.0
	B	F	83.3	16.7	-	-					
	C	G	92.9	7.1	-	-					
	D	H	84.6	15.4	-	-					
Petiole	A	E	7.1	35.7	57.1	-	25.0	10.0	65.0	65.0	76.9
	B	F	84.6	15.4	-	-					
	C	G	85.7	14.3	-	-					
Leaf	A	E	100.0		-	-					

NR = No response   CP = Callus proliferation

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pigmentation.

Petiole - Response of petiole explants was good in all the four media and 23.3% to 100% explants developed into callus except that the older explants did not respond to 'D' media. Callus growth was slow to medium and friable in nature. About half of the calli developed green pigmentation

Hypocotyl - Response of hypocotyl explants excised at young stage was good on all the four media. In general, the younger explants responded better than old. 'A' and 'B' media were better as compared to 'C' and 'D' for callusing for old explants. Whereas, in young explants all the 4 media combinations were almost equally effective for callus induction. Callus thus formed were mostly friable in nature, slow to medium growing and green in colour.

Collar - The response of collar explants from young seedling was best on 'B' media and 60% explants developed into callus. On 'A" and 'C' media also 23.3% to 26.7% explants developed into callus but explants did not respond to 'D' media. Calli were friable in nature and slow to medium growing. Callus developed on 'A' media was green whereas that on 'B' and 'C' media was yellow in colour.

Collar explants excised from old seedling did not respond for callusing on any of the four media

Organogenesis - The calli developed from all the explant-media combinations were subjected to sub-culture in shooting media. The response of young explant derived callus was very poor to organogenesis. Only callus proliferation and necrosis of tissue was observed (Table 4.22).

Green globular embryoid like structure were observed in a few cases in collar, hypocotyl and petiole derived calli, although their further development was restricted. Browning of these structure was observed after few days.

Response of shoot initiation was very good in old explant derived callus from hypocotyl and petiole. In both these cases, more than 50% calli induced shoot formation in 'A' media derived callus. The shoots got induced in the callus which were obtained from hypocotyl and petiole and sub culture on 'E' media. Root

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development could also be achieved by sub culturing in root inducing 'RL' media. Response of petiole derived shoots for root induction was better (65%) than hypocotyl derived shoots (33.3%). More than 75% success was achieved in field transfer (Table 4.24).

4.2.4. *T. alexandrinum* genotype 1 - 90P

The seeds of *T. alexandrinum* (genotype 1-90 P) were germinated on MS media without any hormonal supplementation. 95% seeds germinated within 3-4 days and attained an height of 5.6 cm within 8-10 days. The explants like collar, hypocotyl, petiole and leaf were excised at two stages of seedling growth *i.e.* young (10 days old) and old (30 days old)and placed on different media preparation for callus induction (Table 4.25 and 4.27).

Leaf- The leaf explants from young seedlings responded (50 to 70%) for callusing in all the four media 'A', 'B', 'C' and 'D' whereas, 100% and 70% old explants developed callus in 'B' and 'C' media respectively. All the callus grown on either media was compact in nature and slow to medium growing. Most of the calli developed pigmentation and were green in colour.

Petiole- The response of old seedling explants was better in 'B' and 'C' media as compared to young explants but the old explants showed no response in 'A;' and 'D' media as against good response of young explants (36.7 % and 80% in 'D' and 'A' media respectively). All the calli developed on either media were friable except few cultures developed on 'C' media which were compact in nature. In all the cultures the growth of callus was from slow to medium. Invariably all the calli developed green pigmentation. However, the colour varied from light green to dark green.

Hypocotyl - The explants from old seedlings did not respond to any of four media and all the explants turned brown after 10-15 days.

Young seedling explants responded positively and almost all the explants in 'A' and 'C' media and nearly half of the explants in 'B' and 'D' media developed callus. In all the cases, the calli thus formed were friable in nature. Growth of the

Table 4.25 : Callusing response of *T.alexandrinum* explants in different medium

Genotype - 1-90P   Age of explant – Young seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	28	25.3±10.70	Compact	Yellow	Slow
	B	25	0	-	-	-
	C	25	0	-	-	-
	D	28	0	-	-	-
Hypocotyl	A	30	90.0±9.13	Friable	Yellow	Slow
	B	30	50.0±11.79	Friable	Yellow, Green	Slow, Medium
	C	30	96.7±7.45	Friable	Yellow, Green	Slow
	D	30	40.0±25.28	Friable	Yellow	High
Petiole	A	30	80.0±13.94	Friable	Green	Medium
	B	30	30.0±13.94	Friable	Green	Slow
	C	30	70.0±13.94	Compact, Friable	Yellow, Green	Slow, Medium
	D	30	36.7±7.46	Friable	Pale Green	High
Leaf	A	30	50.0±16.67	Compact	Green	Slow
	B	30	50.0±11.79	Compact, nodular	Hyaline, Green	Slow
	C	30	70.0±21.73	Compact	Yellow, Green	Slow, Medium
	D	30	60.0±9.13	Compact	Yellow	Medium

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	71443.66	4762.911	29.1845**
A. Media	3	14277.66	4759.221	29.1619**
B. Explant	3	46943.45	15647.82	95.8813**
AxB	9	10222.54	1135.838	6.9598**
Within sub group (error)	64	10444.8	163.1999	

** = Significant at 1% level.

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Table 4.26 : Subculturing response in _T. alexandrinum_

Genotype – 1-90 P   Age of explant – Young seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					Response of shoots to rooting media (RL) on subculture (%)		
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting
Collar	A	E	90.0	10.0	-	-			
Hypocotyl	A	E	20.0	20.0	60.0	-	60.0	40.0	-
	B	F	50.0	50.0	-	-			
	C	G	50.0	50.0	-	-			
	D	H	20.0	80.0	-	-			
Petiole	A	E	10.0	90.0	-	-			
	B	F	20.0	80.0	-	-			
	C	G	30.0	70.0	-	-			
	D	H	-	100.0	-	-			
Leaf	A	E	100.0	-	-	-			
	B	F	100.0	-	-	-			
	C	G	-	100.0	-	-			
	D	H	-	100.0	-	-			

NR = No response   CP = Callus proliferation

Table 4.27: Callusing response of *T. alexandrinum* explants in different medium

Genotype - 1-90P   Age of explant – Old Seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	13.3±13.94	Compact	Yellow	Slow
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-
Hypocotyl	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-
Petiole	A	30	0	-	-	-
	B	30	50.0±11.79	Friable	Green	Medium
	C	30	100	Friable	Pale Green	Slow
	D	30	0	-	-	-
Leaf	A	30	0	-	-	-
	B	30	100	Compact	Yellow	Slow
	C	30	70.0±7.45	Compact	Green	Slow
	D	30	0	-	-	-

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	103167.6	6877.84	282.9821**
A. Media	3	29750.3167	9916.7722	408.016**
B. Explant	3	29750.3167	9916.7722	408.016**
AxB	9	43666.9667	4851.8852	199.6261**
Within sub group (error)	64	1555.5112	24.3049	

** = Significant at 1% level.

Table 4.28 : Subculturing response in *T. alexandrinum*

Genotype – 1-90 P   Age of explant – Old seedling

| Explant source | Callusing media | Response of callus to shooting media on sub-culture (%) | | | | |
		Media	NR	CP	Shooting	Rooting
Collar	A	E	83.3	16.7	-	-
Petiole	B	F	11.1	88.9	-	-
	C	G	20.0	80.0	-	-
Leaf	B	F	15.4	84.6	-	-
	C	G	85.7	14.3	-	-

NR = No response   CP = Callus proliferation

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callus was very slow in 'A', 'B' and 'C' media whereas it developed fast in media 'D'. Callus growing on 'A' and 'D' media did not develop chlorophyll pigmentation and remained yellow to pale yellow in colour whereas nearly half of the calli growing on 'B' and 'C' media developed green colour after 2-3 weeks of culture.

Collar- The explants placed on 'A' media induced swelling after 5-7 days and gradually transformed in callus. The response of old explants was less (13.3%) as compared to young seedling explants (25.3%). Irrespective of age of seedling the collar explants did not respond to media 'B', 'C' and 'D'. Callus developed on A media was compact, yellow and slow growing in both cases.

Organogenesis- The calli obtained were sub cultured on four shoot inducing media 'E', 'F', 'G' and 'H'. Response of young hypocotyl explants derived calli developed on 'A' media and sub-cultured on 'E' media was very good. 60% of the calli responded positively for shoot initiation and multiplication. Shoots were initiated 20-23 days after sub-culturing. Shoots were then split and sub-cultured on 'RL' media for root initiation (Table 4.26). However, no root induction was observed and shoots turned brown which resulted in gradual drying up of whole shoots. Repeated sub-subculturing also could not induce root formation.

Calli induced from leaf, petiole and collar showed callus proliferation only and no organogenetic response was noticed.

The calli derived from old explants were not responsive to organogenesis and showed callus proliferation only which did not show differentiation even after sub-culturing 2-3 times (Table 4.28).

4.2.5.*T. alexandrinum* genotype 9-90 N

Seeds of *T. alexandrinum* (genotype 9-90 N) were germinated on MS media without any hormonal supplementation and explants from collar, hypocotyl, petiole and leaf were excised from seedlings at two growth stages *i.e.* young (10 days old) and old (30 days old) and used for callus induction in various media preparations (Tables 4.29 and 4.31, plate 9).

Table 4.29 :Callusing response of *T. alexandrinum* **explants in different medium**

Genotype - 9-90-N   Age of explant – Young seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	0	-	-	-
	B	30	23.3+9.13	Friable	Green	Slow
	C	30	13.3+7.45	Friable	Yellow	Slow
	D	30	40.0+9.13	Friable	Green	Slow
Hypocotyl	A	30	50.0+23.57	Friable	Green	Medium
	B	30	70.0+24.72	Friable, Compact	Green	High
	C	30	43.3+30.28	Friable, compact	Green	High
	D	30	66.7+23.57	Friable	Green	Medium
Petiole	A	30	43.3+19.00	Friable	Green	Slow
	B	30	40.0+14.91	Friable, Compact	Green	Medium
	C	30	23.3+9.13	Friable	Green	Slow
	D	30	60.0+25.28	Friable	Green	Medium
Leaf	A	30	33.3+23.57	Compact	Green	Slow
	B	30	76.7+9.13	Compact	Green	Slow
	C	30	43.3+9.13	Compact	Yellow	Medium
	D	30	46.7+18.26	Compact	Green	Slow

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	31874.28	2124.952	6.5106**
A. Media	3	9402.847	3134.282	9.6031**
B. Explant	3	16513.420	5504.473	16.8651**
AxB	9	5958.008	662.001	2.0283
Within sub group (error)	64	20888.44	326.382	

** = Significant at 1% level.

Table 4.30 : Subculturing response in *T. alexandrinum*

Genotype – 9-90N   Age of explant – Young seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					Response of shoots to rooting media (RL) on subculture (%)			Complete plantlets (%)	Field Transfer (%)
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting		
Collar	B	F	72.7	27.3	-	-					
	C	G	27.3	72.7	-	-					
	D	H	25.0	75.0	-	-					
Hypocotyl	A	E	10.0	40.0	50.0	-	20.0	26.7	53.3	53.3	20
	B	F	20.0	80.0*	-	-					
	C	G	33.3	66.7*	-	-					
	D	H	18.2	54.5	27.3	-	80.0	20.0	-		
Petiole	A	E	100.0	-	-	-					
	B	F	66.7	33.3	-	-					
	C	G	30.0	70.0	-	-					
	D	H	30.0	70.0	-	-					
Leaf	A	E	83.3	16.7	-	-					
	B	F	100.0	-	-	-					
	C	G	100.0	-	-	-					
	D	H	23.1	76.9	-	-					

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

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Table 4.31: Callusing response of *T. alexandrinum* explants in different medium

Genotype - 9-90N   Age of explant – Old seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	50.0±11.79	Compact	Hyaline	Slow
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-
Hypocotyl	A	30	0	-	-	-
	B	30	16.7±11.79	Friable	Green	Slow
	C	30	33.3±16.67	Friable	Green	Slow
	D	30	0	-	-	-
Petiole	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	56.7±9.13	Friable, compact	Hyaline, Green	Slow
	D	30	16.7±11.79	Friable, Compact	Green	Slow, Medium
Leaf	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	27500.92	1833.394	37.7166**
A. Media	3	4555.611	1518.537	31.2394**
B. Explant	3	3583.567	1194.522	24.5737**
AxB	9	19361.74	2151.304	44.5737**
Within sub group (error)	64	3111.02	48.6097	

** = Significant at 1% level

Table 4.32 : Subculturing response in *T. alexandrinum*

Genotype – 9-90 N   Age of explant – Old seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					Response of shoots to rooting media (RL) on subculture (%)		
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting
Collar	A	E	100.0	-	-	-			
Hypocotyl	B	F	36.4	36.4	27.2	-	75.0	25.0	-
	C	G	50.0	33.3	16.7	-	62.5	37.5	-
Petiole	C	G	76.9	23.1	-	-			
	D	H	16.7	83.3	-	-			

NR = No response   CP = Callus proliferation

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Leaf - Response of young explants was very good and 33 to 76.7% explants developed callus against no response of old explants. Leaf explants invariably developed in compact and green callus except from 'C' media which did not develop green pigmentation. Growth rate was slow in green calli.

Petiole - Young explants showed moderately good response to callus induction in all the 4 media. However, best response was observed in 'D' media followed by 40 and 43% in 'B' and 'A' media respectively.

Old explants showed no response in 'A' and 'B' media and poor (16.7%) in 'D' media. Whereas, good response (56.7%) was observed in 'C' media. In all the cases, calli were friable/ compact, hyaline in colour initially but developed pigmentation after 15-20 days. The growth rate was initially slow and callus initiation could be observed only after 10-12 days but later on growth rate of callus was medium.

Hypocotyl - Young explants responded well on all the four media. Most of the explants showed swelling in first 15-20 days whereas a few explants on 'B' and 'C' media showed very quick response and friable green callus formation was observed within 15-20 days followed with prolific callus growth in next 10-15 days. Most of the calli formed were friable in nature and green in colour. The growth varied from medium to high.

Few explants from old seedlings showed callus induction on 'B' and C media (16.7 and 33.3% respectively) only whereas there was no response in other two 'A', and 'D' media. Callus formed was bright green and shooting initiated after 30 days in callusing media itself.

Collar: Callusing response of old explants in 'A' media was 50% whereas, no positive response was observed in the rest 3 media. Callus was compact, hyaline and slow growing.

Response of young explants was not very good and only 13 to 40% explants showed callusing in 'B', 'C' and 'D' media. All such calli were friable in nature and majority of them developed green colour while a few remained pale

yellow in colour. The growth of callus was also very slow. Swelling was induced in 15-20 days and friable bright green callus was formed in 30-35 days. No response of young seedling was observed in 'A' media

Organogenesis - Calli obtained from different explant-media combinations were tried for shoot induction by sub culturing in shoot induction media. Young stage derived calli from leaf, petiole, collar showed only callus proliferation and no organogenetic response was observed. In 'F' and 'G' media cultures of hypocotyl derived calli embryoid like green lobes developed but no shooting was observed in such cultures.

Calli derived from young hypocotyl explants on sub-culture responded positively for shoot induction. The shoots were multiplied by one more round of sub-culturing in same media. Shoots were split and transferred to 'RL' media. No root induction was observed in shoots derived on 'H' media. Successful root induction was noticed in shoots induced on 'E' media. Success rate of field transfer was also poor in this case. Only 20% plants could survive the ordeal of hardening (Table 4.30).

Response of old stage hypocotyl explants induced calli was also good for shoot induction. Shooting was initiated after 20-25 days of subculturing in 'G' shooting media whereas shooting could be induced in 'F' media only after 40 days when sub cultured for second time in same media However, rooting could not be observed in these shoots even after 2 rounds of sub-culturing in 'RL' media. Shoot multiplication was however observed in some of the tubes (Table 4.32).

In some cases, shooting induced in callusing media itself such shoots were transferred to 'F' media showed growth of shoots. These shoots did not respond to 'RL' media for root induction even after 2-3 subculturing in root inducing media.

4.2.6. *T. alexandrinum* genotype 3-90H

The seeds of *T. alexandrinum* genotype 3-90 H were germinated on MS media devoid of any growth hormone and explants from collar, hypocotyl, petiole and leaves were excised from 10 and 30 days old seedlings and cultured on 'A',

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Table 4.33: Callusing response of *T. alexandrinum* explants in different medium

Genotype - 3-90 H   Age of explant – Young seedling

Explant Source	Media	No. of explants	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	23.3±9.13	Friable	Green	Medium
	D	30	0	-	-	-
Hypocotyl	A	30	36.7±13.94	Friable	Green	Medium
	B	30	36.7±21.73	Friable	Green	High
	C	30	43.3±25.28	Friable	Green	Medium
	D	30	40.0±14.91	Friable	Yellow, Green	High
Petiole	A	30	50.0+0.00	Friable	Green	Medium
	B	30	50.0±11.79	Friable	Green	Slow
	C	30	50.0±23.57	Friable	Yellow, Green	Slow
	D	30	46.7±18.26	Friable	Green	Medium
Leaf	A	30	20.0±13.94	Friable	Green	Slow
	B	30	60.0±9.13	Compact	Yellow	Slow
	C	30	40.0±14.91	Friable	Hyaline	Slow
	D	30	46.7±13.94	Compact	Hyaline	Slow

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	29524.51	1968.3	10.401**
A. Media	3	5760.68	1920.227	10.147**
B. Explant	3	17732.52	5910.841	31.236**
AxB	9	6031.304	670.145	3.541**
Within sub group (error)	64	12110.96	189.234	

** = Significant at 1% level

Table 4.34 : Subculturing response in *T. alexandrinum*

Genotype – 3-90 H   Age of explant – Young seedling

Explant source	Callusing media	Response of callus to shooting media on subculture (%)					Response of shoots to rooting media (RL) on subculture (%)		
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting
Collar	C	G	8.3	91.7*	-	-			-
Hypocotyl	A	E	20.0	40.0	40.0	-	57.1	42.9	
	B	F	14.3	85.7	-	-			
	C	G	37.5	62.5	-	-			
	D	H	13.3	86.7	-	-			
Petiole	A	E	35.7	64.3	-	-			
	B	F	23.1	76.9	-	-			
	C	G	40.0	60.0	-	-			
	D	H	16.7	83.3*	-	-			
Leaf	A	E	27.3	72.7	-	-			
	B	F	23.1	76.9	-	-			
	C	G	33.3	66.7	-	-			
	D	H	53.3	46.7	-	-			

NR = No response   CP = Callus proliferation   * = Somatic embryoids also formed in some cases

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Table 4.35: Callusing response of *T. alexandrinum* explants in different medium

Genotype - 3-90 H   Age of explant – Old seedling

Explant Source	Media	No. of explants cultured	% callus induction	Callus characteristics		
				Nature	Colour	Growth rate
Collar	A	30	50.0±11.79	Compact	Yellow	Slow
	B	30	100	Friable	Hyaline	Slow
	C	30	100	Friable	Hyaline	Slow
	D	30	0	-	-	-
Hypocotyl	A	30	100	Friable	Green	Medium
	B	30	50.0±11.79	Friable, nodular	Green	Medium
	C	30	56.7±9.13	Friable	Green	Medium
	D	30	80.0±13.94	Friable	Pale Green	Medium
Petiole	A	30	100	Friable	Pale Green	Slow
	B	30	66.7±26.35	Friable	Green	Medium
	C	30	66.7±31.18	Friable	Pale Green	Medium
	D	30	0	-	-	-
Leaf	A	30	0	-	-	-
	B	30	0	-	-	-
	C	30	0	-	-	-
	D	30	0	-	-	-

Anova table

Source of Variation	Df	SS	MS	F
Sub groups	15	132218.8	8814.584	63.463**
A. Media	3	21871.6	7290.534	52.4899**
B. Explant	3	63621.79	21207.26	152.687**
AxB	9	46725.38	5191.709	37.379**
Within sub group (error)	64	8889.211	138.8939	

** = Significant at 1% level

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Table 4.36 : Subculturing response in *T. alexandrinum*
Genotype – 3-90 H   Age of explant – Old seedling

Explant source	Callusing media	Response of callus to shooting media on sub-culture (%)					Response of shoots to rooting media (RL) on subculture (%)		
		Media	NR	CP	Shooting	Rooting	NR	Shooting	Rooting
Collar	A	E	50.0	50.0	-	-			
	B	F	36.4	63.6	-	-			
	C	G	46.7	53.3	-	-			
Hypocotyl	A	E	64.3	35.7	-	-			
	B	F	57.1	42.9	-	-			
	C	G	41.7	58.3*	-	-			
	D	H	-	46.7	53.3	-	61.5	38.5	-
Petiole	A	E	41.7	58.3	-	-			
	B	F	38.5	61.5	-	-			
	C	G	73.3	26.7	-	-			

NR = No response   CP = Callus proliferation
* = Somatic embryoids also formed in some cases

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'B', 'C' and 'D' media. Response towards callus induction and nature of calli formed is presented in tables 4.33 and 4.35.

Leaf - Response of leaf explants excised from young seedling was low to moderate and 20 to 60% explants developed in callus whereas leaves collected from old seedling did not respond to any of the four media tried. Growth of calli was very slow and hyaline or pale yellow calli were formed except for a few calli which developed green colour on 'A' media. The calli on 'A' and 'C' media were friable in nature whereas those developed on 'B' and 'D' media were compact.

Petiole - Response of young petiole explants was good on all the four media wherein 46.7 to 50% explants developed into calli. The calli were slow to medium in growth and friable in nature. All the calli developed green pigmentation 15-20 days after inoculation.

Response of old explants was very good in 'A', 'B' and 'C' media (66.7 to 100% callus induction) whereas no calli were induced in 'D' media. Invariably all the calli formed were friable in nature. Growth of callus was slow to medium.

Hypocotyl - Response of hypocotyl explants excised at both growth stages was moderate to good on all the four media and 37 to 100% explants developed into calli. All the calli were friable in nature and green in colour. Growth of calli was medium to high.

Collar - Response of explants from young seedling was not good and only 23.3% explants developed into callus on 'C' media whereas no callusing response was found in other three media ('A', 'B', 'D'). The callus developed chlorophyll pigmentation and turned green after 20-25 days.

Response of explants from old seedlings was good and 50 to 100% explants developed into callus in 'A', 'B' and 'C' media. Calli thus formed were friable in nature and growth varied from slow to medium. A few calli developed on 'A' media from old explants were compact, yellow and slow in growth and none of calli developed green pigmentation.

Organogenesis - Callus developed from different explants was sub cultured on

four media 'E', 'F', 'G' and 'H' after 30-35 days of inoculation in callus inducing media. Callus derived from young explants showed vigorous callus proliferation on four sub culture media and no response of shooting or organogenesis was observed except in hypocotyl induced callus developed on 'A' media and sub-cultured in 'E' media. In this case 4 out of 10 tubes cultured showed emergence of shoots 15-20 days after sub-culturing (table 4.34). In a few cultures of young collar explants, developed on 'C' media and sub-cultured in 'G' media green globular embryoid like structures were noticed.

Response of calli derived from old explants was similar. No organogenic response was observed in petiole and collar derived calli. However, hypocotyl derived calli in 'D' media, sub-cultured in 'H' media showed shoot emergence in 12-15 days (table 4.36). The shoots were multiplied by one more round of sub-culture in 'H' media. There was profuse leaf development and the shoots were dwarf in nature. The multiplied shoots were split and sub-cultured in root inducing 'RL' media. However, rooting could not be induced even after repeated sub-culturing in 'RL' media. The lower cut part of the shoot turned brown and gradually the whole shoot dried up in different tubes in 25-45 days after initially put in 'RL' media.

4.3. Characterization of calli and regenerants

4.3.1. Morphological studies

4.3.1.1. *T. alexandrinum* regenerants :

The plants of *T.alexandrinum* genotype transferred to field initially developed reddish green pigmentation. The leaves and stem were reddish green in colour but later on, after 15-20 days, new branches started growing from the axillary parts. In many cases the plants transferred to field showed initiation of flowering which was as such not matching to the flowering time of mother plant. Plants flowered in January itself. It was also noticed that the plants even after flowering continued to give out branches. Thus the regenerant remained in vegetative phase for some more period and then second flush of flowering was noticed. The seed set in first flush of flowering i.e. just after transplant was less (5-7 seeds per inflorescence) as compared to good seed set in second flush (30-55

seeds per inflorescence). In one plant, the secondary branches were found emerging on opposite direction from the same node, whereas in the mother plant, the leaves were alternately arranged and small secondary branches emerged from axils of these nodes. Other morphological traits were similar to mother plants. The detailed data on morphological attributes for comparison with mother plant was not recorded as there was difference in the age of plant regenerated and that growing in the field.

4.3.1.2. *T. resupinatum* regenerants :

The regenerant of *T. resupinatum* transferred to field were morphologically similar to the mother plant. The difference in quantitative traits such as leaf length, leaf width, plant height, stem girth were due to difference in age of the plants and hence were not recorded. In this species also *in vitro* flowering of regenerant was obtained and seeds in first flush of lowering was less which recovered in second flush and 10-20 seeds per inflorescence were harvested.

4.3.2. Isozymic studies

4.3.2.1. Isozyme banding pattern in regenerant of *T. resupinatum*

In –vitro regenerant and the mother plant of *T. resupinatum* were compared by studying the isozyme profiles of five enzymes. A few other accessions of *T. resupinatum* were also included to get the comparative profile of the variation found in the species (plate 11,12).

Esterase : A total of six bands were observed for Esterase isozyme at 0.58, 0.61, 0.63, 0.72, 0.77 and 0.90 points of relative mobility. In tissue culture regenerated plant only two bands were present (Band 4 and 6). The same bands were observed in the mother plant too. Esterase isozyme banding pattern in other accessions of *T. resupinatum* differed and three or more bands were seen in other accessions. Band number 6 was found to be common in all the samples (Table 4.37).

Super Oxide Desmutase : Three bands of SOD isozyme were observed among different *T resupinatum* samples. These bands were at 0.52, 0.73 and 0.87 RM value points. Band no.2 was absent in regenerant as compared to its mother plant. Intensity of Band 1 also varied among different plants. This band was quite

prominent in the regenerated plant as compared to its mother plant. No variation was observed among different accessions of the species.

Acid Phosphatase : ACP isozyme pattern among different accessions and regenerant revealed the presence of total three bands. These bands were marked at 0.45, 0.58 and 0.73 RM values. Band 1 and 2 were invariably present in all the accessions and the regenerant. There was no difference for banding pattern between mother plant and regenerant.

Glutamate Oxalo-Acetate Transferase : Considerable variation for isozyme pattern was observed between regenerant and its mother plant. Total five bands were observed in mother plant in contrast to only two bands present in the regenerant. Band 1, 2, 3, 4 and 5 were present in mother plant and Band 3 and 4 were noticed in regenerant. There was no variation in different accessions of the species and all the accessions share five bands. These bands were found at 0.31, 0.33, 0.39, 0.44 and 0.47 RM points in the gel.

Peroxidase : Peroxidase banding was also recorded in *T. resupinatum* mother plant and the regenerant. No variation for anodal peroxidase banding pattern was observed and two bands were commonly present in all the accessions as well as mother and regenerant plant. Towards cathodal end contrasting view was observed between mother plant and regenerant. Band no. 1,2,4 were present in regenerant whereas Bands 3,5 were found in mother plant.

Similarity index : Similarity matrix analysis using Dice's co-efficient showed that the regenerant is only 69% similar to the mother plant. The mother plant and other accessions of the *T. resupinatum* used in this study showed 82.4 to 91.4% similarity among themselves. (Table 4.38)

The regenerant showed a totally different isozyme pattern indicating thereby development of a new somaclonal variant plant. Dendrogram based on cluster analysis of isozyme data showing genetic relatedness between different accessions, mother plant and regenerant of *T. resupinatum* has been presented in fig. 5.16.

Table 4.37 : Isozyme banding pattern in regenerated and mother plant along with other genotypes of *T. resupinatum*

Genotype	Esterase	SOD	Peroxidase		ACP	GOT
			Anodal	Cathodal		
SH- 98-34	3, 5, 6	1, 2, 3	1, 2	3,4	1, 2	1, 2, 3, 4, 5
SH- 98-34-1	3, 5, 6	1, 2, 3	1, 2	2,3,4,5	1, 2	1, 2, 3, 4, 5
SH- 98-15	3, 5, 6	1, 2, 3	1, 2	2,3,4,5	1, 2, 3	1, 2, 3, 4, 5
SH- 97-BS1	4, 5, 6	1, 2, 3	1, 2	2,3,5	1, 2, 3	1, 2, 3, 4, 5
SH- 97-BS2	1, 2, 5, 6	1, 2, 3	1, 2	3,4	1, 2	1, 2, 3, 4, 5
SH-98-49 (Mother plant)	4, 6	1, 2, 3	1, 2	3,5	1, 2	1, 2, 3, 4, 5
SH-98-49 (Regenerated plant)	4, 6	1, 3	1, 2	1,2,4	1, 2	3, 4

Table 4.38. : Similarity matrix based on isozyme banding pattern in mother plant and regenerant along with other accessions of *T. resupinatum*

Genotypes	1	2	3	4	5	6	7
1	1.000						
2	0.944	1.000					
3	0.919	0.974	1.000				
4	0.833	0.895	0.923	1.000			
5	0.914	0.865	0.842	0.811	1.000		
6	0.848	0.857	0.833	0.914	0.824	1.000	
7	0.667	0.688	0.667	0.688	0.645	0.690	1.000

4.3.2.2. Isozyme banding pattern in calli of different age in *T. alexandrinum* :

Calli of different ages developed from JHB 146 and 1-90 P were analyzed for isozyme banding pattern of SOD, ACP, Peroxidase and esterase.

Banding pattern in genotype JHB 146 :

The calli from JHB 146 were collected at 40,60,90 and 300 days of inoculation along with one regenerant at vegetative stage and the other regenerant at *in-vitro* flowering stage. Leaf samples were taken from the regenerant. Green proliferating part of callus and leaf part of regenerant and mother plant were used for extract preparation (plate 13).

No variation was observed for SOD, ACP and esterase isozymes with

presence of 3,3 and 5 bands respectively in all the cases. High degree of variation was observed for peroxidase banding pattern. Towards anodal end, maximum five bands were observed in 40 days old calli but in 300 days old calli as well as in regenerant (both vegetative and flowering stage), the number of anodal bands observed were only 2. In general, the callus possessed more number of bands as compared to regenerant or mother plant. For peroxidase, no variation was observed for regenerant at both vegetative and flowering stage. Towards cathodal end, maximum five bands were observed in flowering regenerant and minimum two bands observed in 300 days old calli. Maximum bands were present in mother plant (table 4.39).

Table 4.39 : Isozyme banding pattern in callus of different ages and in regenerant and flowering regenerant in JHB 146

Plant/callus	Peroxidase		SOD	ACP	Esterase
	Anodal	Cathodal			
Mother plant	3, 5, 7	1, 2, 3, 4,5	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
40 days	2, 4,5,6,7	2, 3, 4,5	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
60 days	1, 4, 5,6	2, 3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
90 days	2, 4,5,7	1, 2, 3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
300 days	2,4,5	1, 2, 3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
Regenerant	4,5	1, 5	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
Flowering Regenerant	4,5	2, 3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5

Banding Pattern in genotype 1-90 P : Calli of tetraploid genotype 1-90 P were collected at 50, 60,75,100 days after explant inoculation, one regenerant having shoot only and the mother plant. Leaf portion of mother plant and regenerant and green proliferating callus of different ages were used for enzyme extraction.

No variation was noticed for SOD and ACP isozyme banding pattern. An additional band no. 6 was found at extreme end in the 100 days old callus which was faster than all other bands observed in different ages of calli as well as mother and regenerant plant (plate 13).

For peroxidase isozyme variation was observed among callus of different ages and mother and regenerant. At anodal end, maximum of 5 bands were observed in calli of 50 days. Band number 7 was absent in shooting regenerant.

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For cathodal peroxidase isozyme, a maximum of 5 bands were recorded. No variation in mother plant and regenerant were observed for cathodal peroxidase. Calli of different ages showed variation and less number of bands (table 4.40).

Table 4.40 : Isozyme banding pattern in callus of different ages and in regenerant and flowering regenerant in genotype 1-90P

Plant/callus	Peroxidase		SOD	ACP	Esterase
	Anodal	Cathodal			
Mother plant	3,5,7	1,2,3,4,5	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
50 days	2,3,5,6,7	2,3,4,5	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
60 days	1,3,5,6	2,3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
75 days	2,4,5,7	2,3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5
100 days	2,4,5	1,2,3,4	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5, 6
Shooting Regenerant	3,5	1,2,3,4,5	1, 2, 3	1, 2, 3	1, 2, 3, 4, 5

4.3.3. Cytological study of callus:

Callus of genotype JHB 146

Hypocotyl derived callus of a diploid genotype JHB 146 growing on … media which were 40, 60, 90 and 300 days old were cytologically examined and frequency of diploid, tetraploid and higher ploidy level cells was measured. Age of callus was determined from the day of explant inoculation. The callus was maintained by repeated sub-culturing in the same media after every 35 days.

In 40 days old callus, 91.8% cells were diploid and only 5.9% and 2.4% cells showed presence of tetraploid and higher ploidy level cells respectively The two cells observed possessed 62 and 60 chromosomes. In 60 days old callus number of diploid cells was reduced to 76.7 % and rise in number of tetraploid and high ploidy level cells was observed. Similar trend was seen in 90 and 300 days old callus which were marked for 30.4% and 36.8% tetraploid cells respectively. Thus, the frequency of diploid cells were found to be gradually decreasing in number and in 300 days old callus only 36.8% cells were diploid in nature whereas, about 63% cells were of polyploid nature (table 4.41, plate 14).

Table 4.41 : Variation in ploidy level in callus tissue of different ages in
genotype JHB 146 of *T. alexandrinum*

Callus age (days)	Cells studied	% cells		
		2x	4x	Higher
40	85	91.8	5.9	2.4
60	90	76.7	15.7	6.7
90	115	52.2	30.4	17.4
300	95	36.8	36.8	26.3

Callus of genotype 1-90-P

Hypocotyl derived callus of the tetraploid genotype 1-90-P growing on ....
media was studied for variation in somatic chromosome numbers. In 40 days old
callus most of cells were tetraploid in nature and only two cells were observed to
possess 64 chromosomes. In 60 days old callus three cells were observed at
diploid level whereas, 71.3% were of tetraploid nature (the level of mother
explants). There was a marked increase in frequency of higher ploidy level cells at
60 days as compared to 40 days old callus. Frequency of higher ploidy level cells
remain almost static between 60 and 90 days old callus followed by a marked
increase in 300 days old callus (table 4.42).

Table 4.42 : Variation in ploidy level in callus tissue of different ages in
genotype 1-90P of *T. alexandrinum*

Callus age (days)	Cells studied	% cells		
		2x	4x	Higher
40	95	-	97.9	2.1
60	87	3	71.3	25.3
90	88	-	73.9	26.1
300	93	-	65.6	34.4

Plate 4. **Different stages of *in vitro* regeneration of plantlets in *T. glomeratum* (EC 401700)**

A. Callus proliferation from petiole explant growing on 'D'media.

B. Shoot initiation in the callus sub cultured on 'E' media.

C. Regeneration of complete plantlets.

Plate : 4
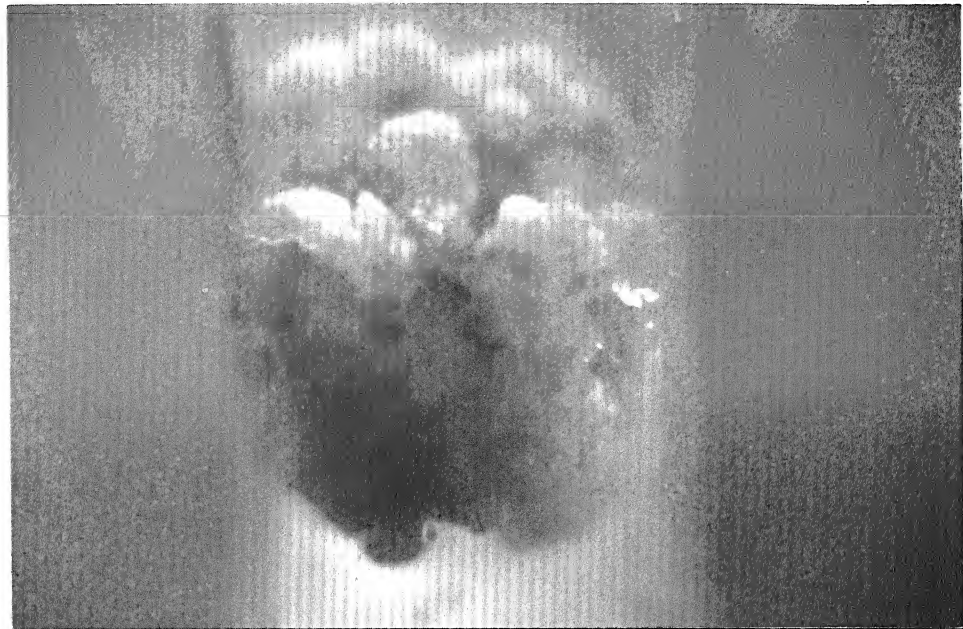
A

B
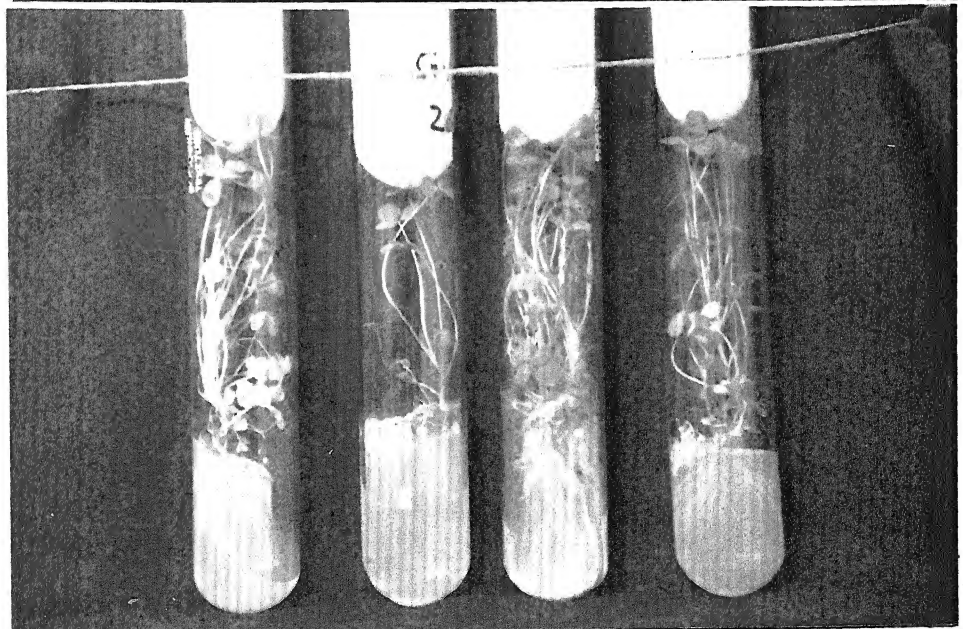
C

Plate 5. Different stages of *in vitro* regeneration of plantlets in *T. apertum* (EC 401712)

A. Callus proliferation from petiole explant growing on 'D' media.

B. Shoot initiation in the callus sub cultured on 'E' media.

Plate : 5

A

B

Plate 6. Different stages of *in vitro* regeneration of plantlets in *T. hybridum* (EC 401702)

A. Callus proliferation from hypocotyl explant growing on 'D'media.
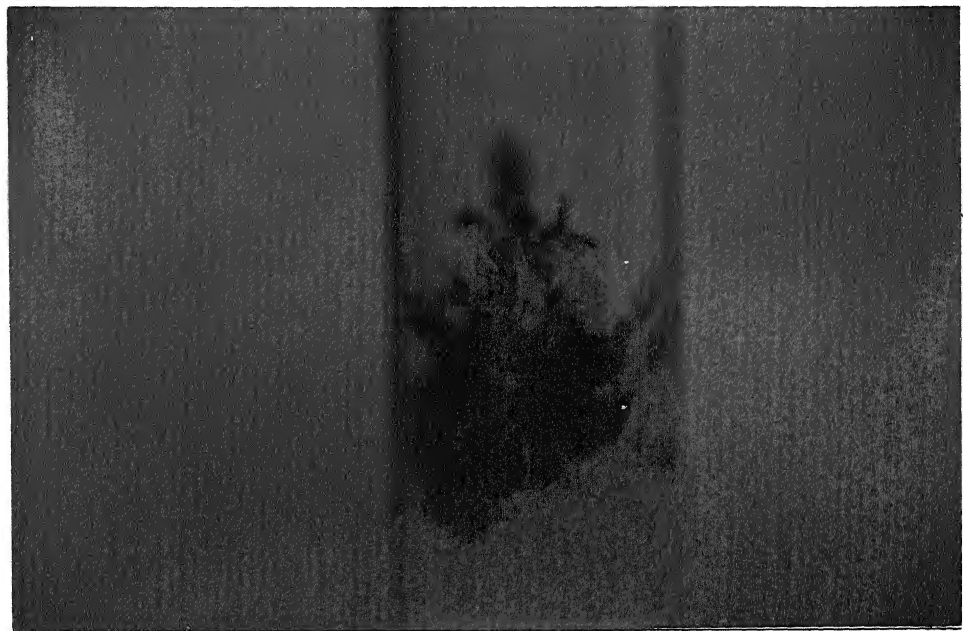
B. Shoot initiation in the callus sub cultured on 'E' media.

C. Shoots in RL media for root initiation.

Plate : 6
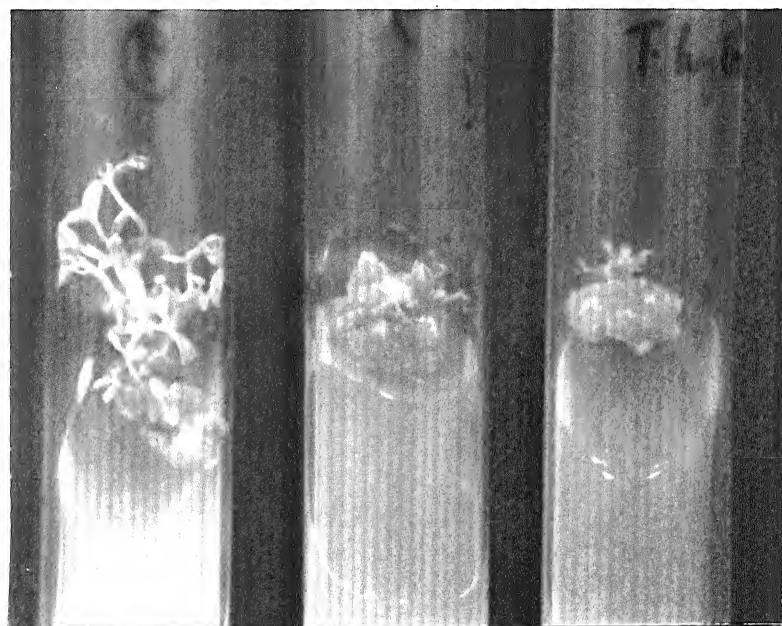
Plate 7. Different stages of *in vitro* regeneration of plantlets in *T. alexandrinum*

A. Complete regenerant following old hypocotyl explant ('B' media →'F' media →'RL' media) in JHB 146.

B. Complete regenerant obtained from old petiole explant ('A' media →'E' media →'RL' media) in FAO-1.

C. Complete regenerant obtained from old petiole explant('A' media →'E' media →'RL' media) in BL 42.

D. and E. *In vitro* flowering observed in the regenerant derived from hypocotyl explant developed on 'A' media →'E' media →'RL' media in BL 42.

Plate : 7
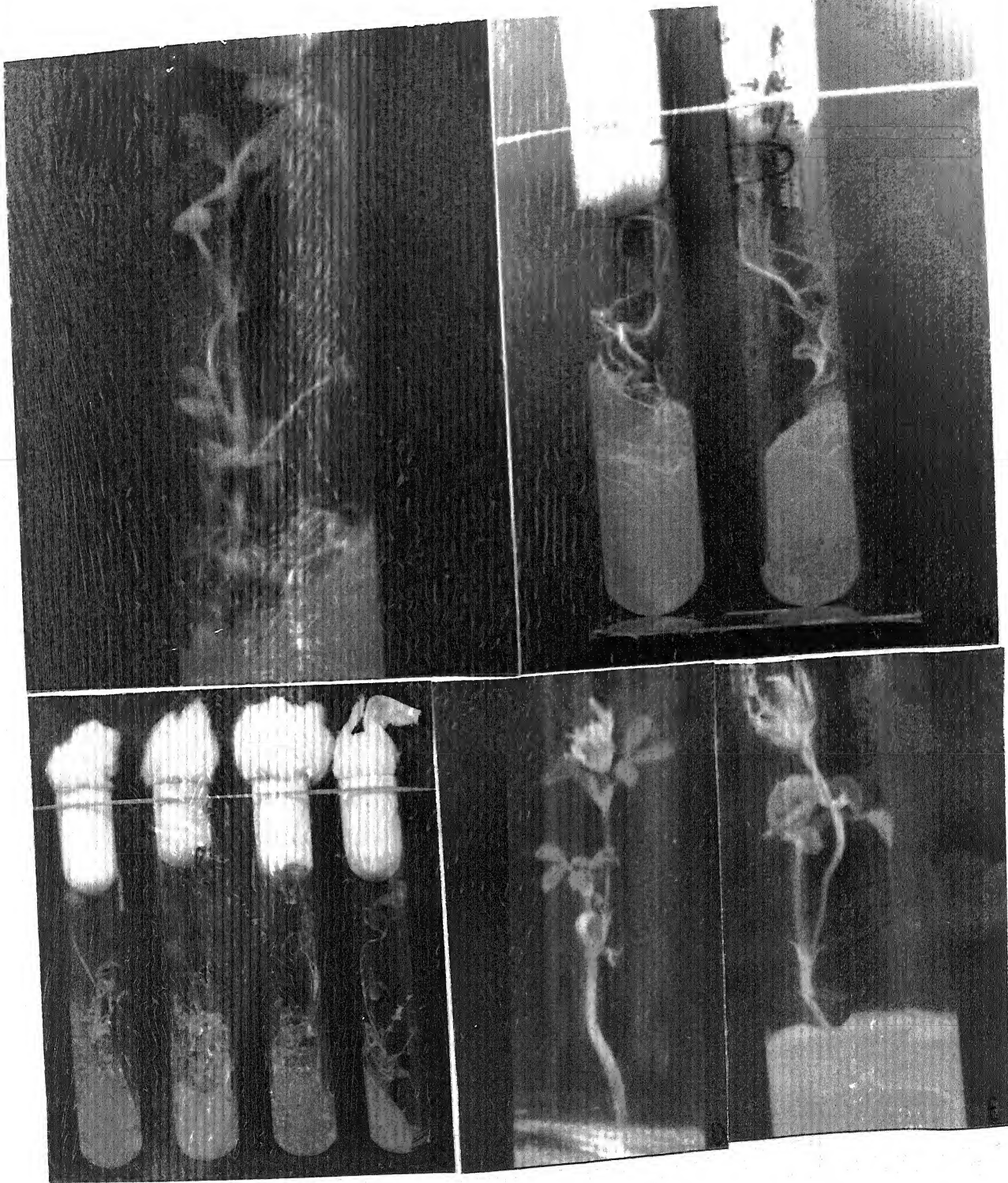
Plate 8. **Different stages of *in vitro* regeneration of plantlets in *T. alexandrinum* (JHB 146)**

A. Shoot emergence in the callus growing on shoot inducing media

B. Multiple shooting in the callus developing on shoot inducing media

C. Multiple shooting in the callus developing on shoot inducing media

D. Callus proliferation.

E. Embryoid formation in the calli

F. Embryogenic calli development.

G. Callus proliferation.
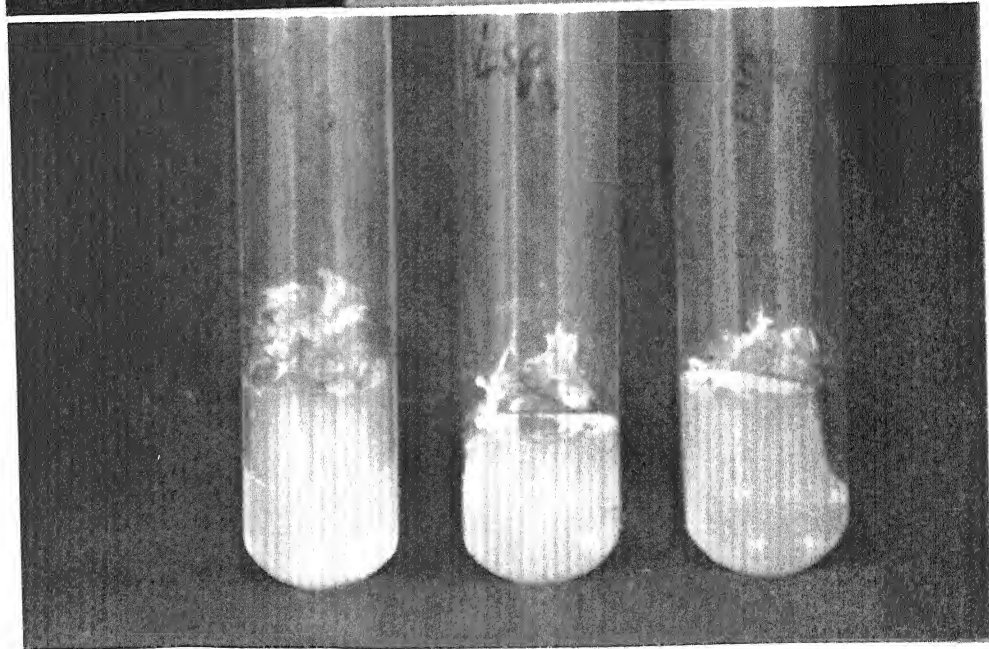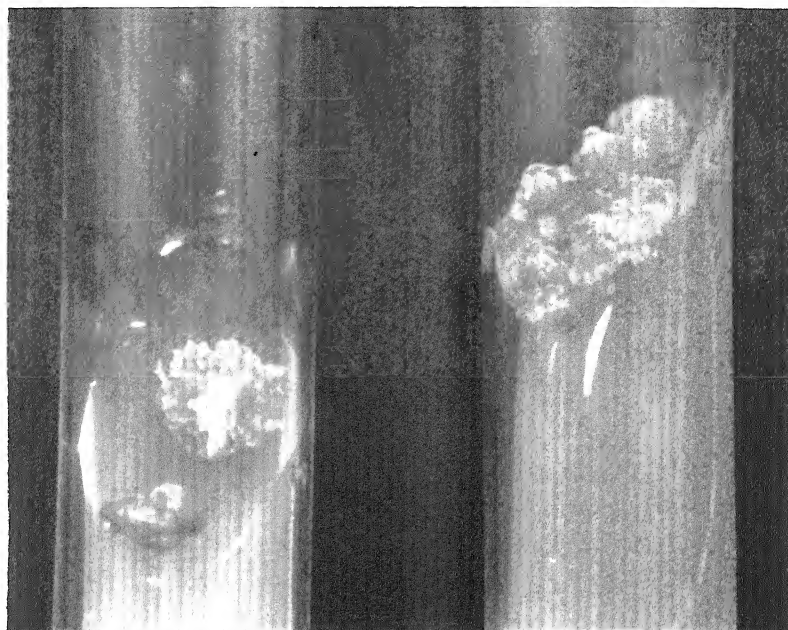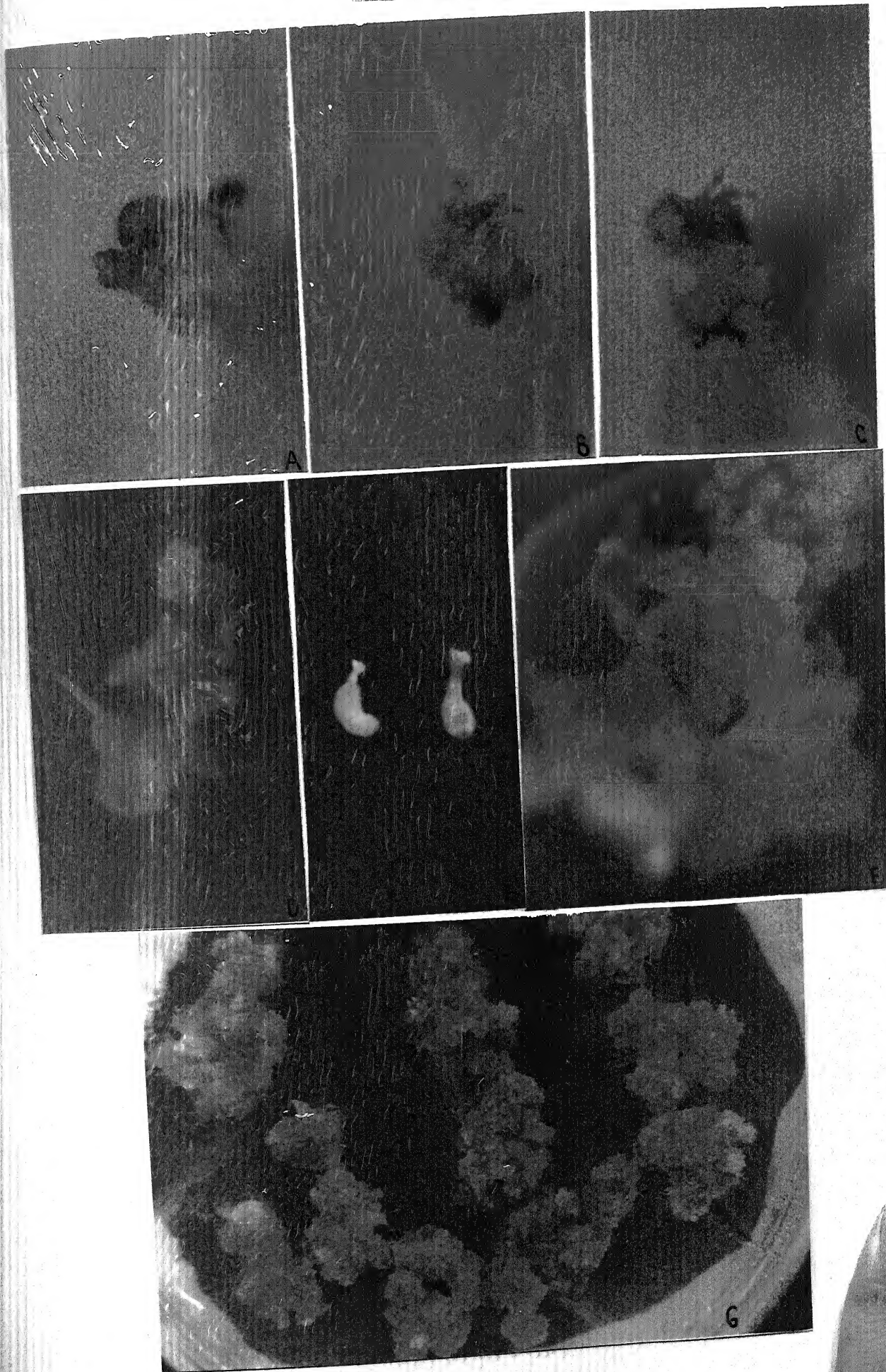
Plate 9. Different stages of *in vitro* regeneration of plantlets in *T. alexandrinum* (9-90 N)

A. Callus proliferation from hypocotyl explant growing on 'A' media.

B. Shoot initiation in the callus sub cultured on 'E' media.

C. Shoots in 'RL' media for root initiation.

D. Multiple shooting in shoots developed on 'E' media.

E. Complete regenerant.

F. Multiple shooting in the callus growing on shoot initiation media 'E'

Plate : 9
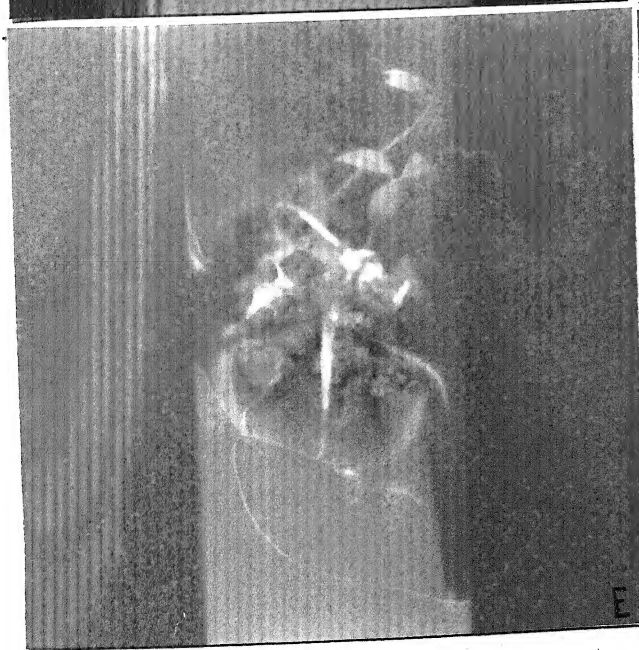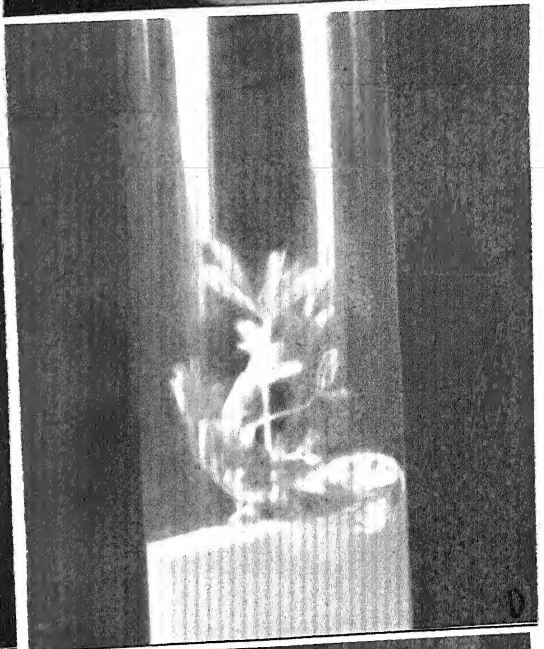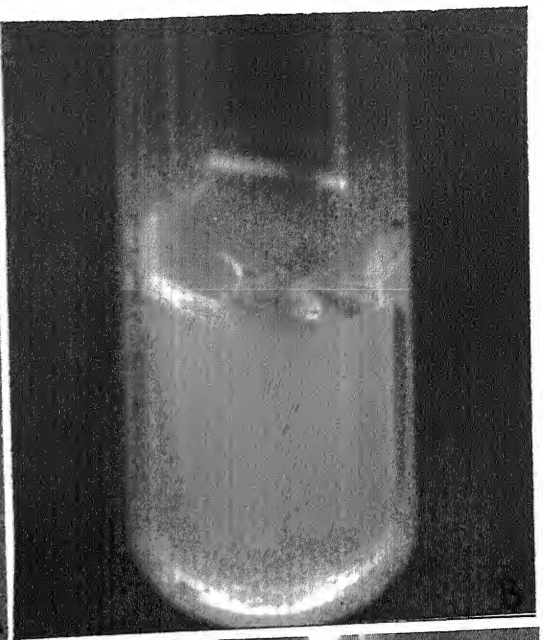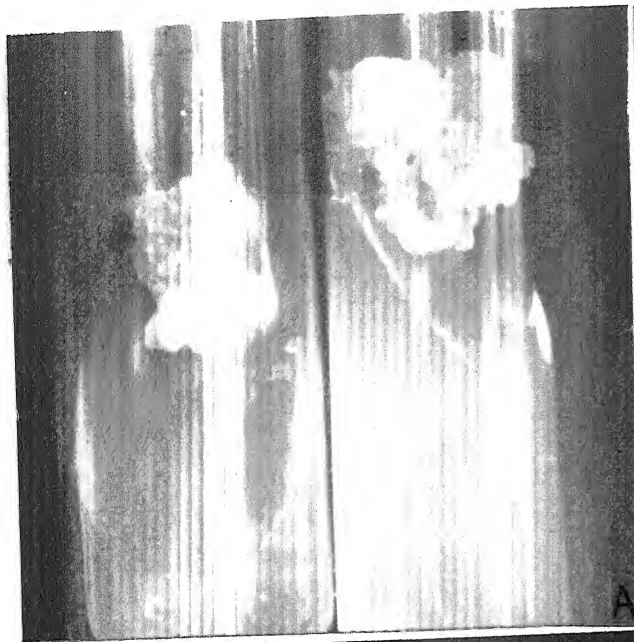
Plate 10. Inoculation of *Rhizobium* in the roots of *in vitro* regenerated plants before transfer to field.

A. Roots of regenerant in Rhizobium culture.

B. Root nodule formation 6 days after inoculation.

C. Regenerant transferred to pot.

Plate : 10
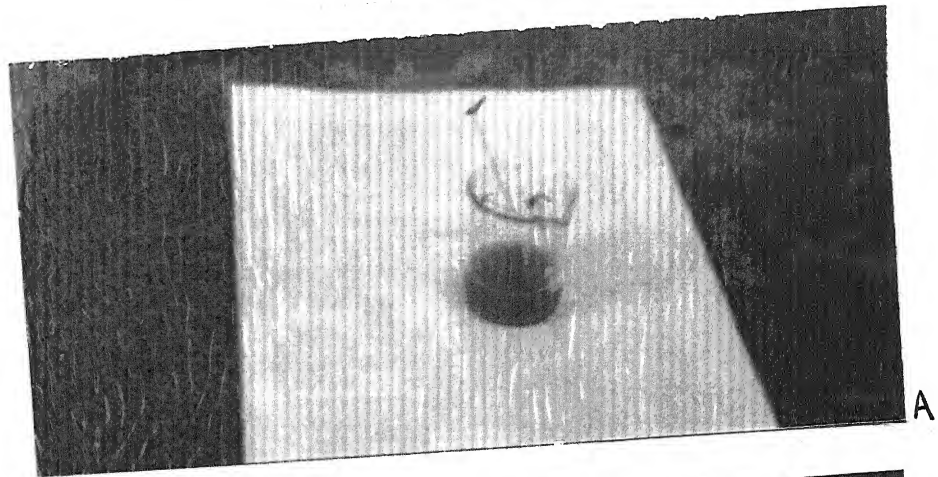
A
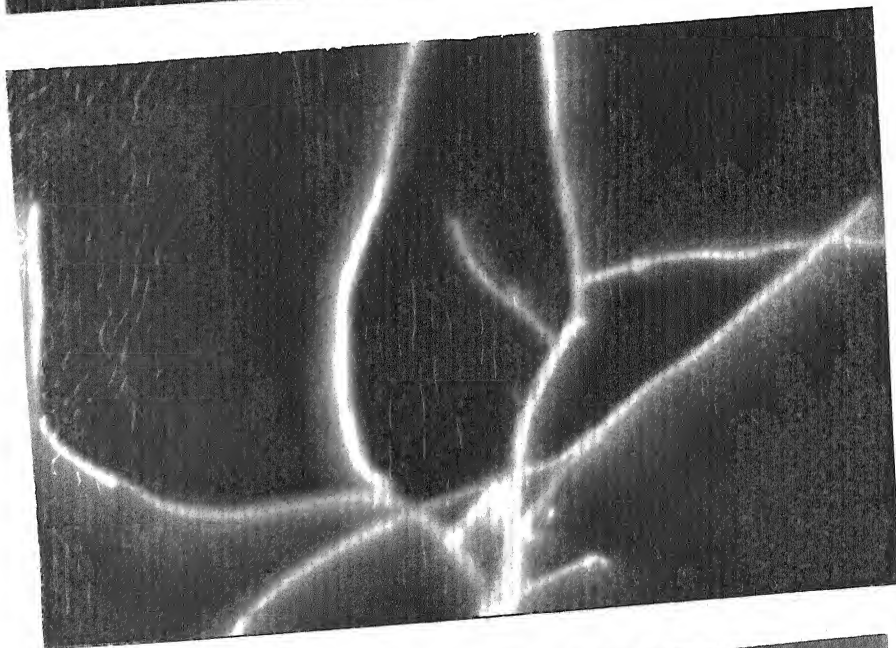
B

C

**Plate 11. Isozyme variation in regenerant and the
mother plant of *T. resupinatum.* (SH 97-49)**

A. Variation for ACP bands: from L to R
Test sample
SH 97- BS1
SH 97- BS2
Test sample
SH 97- 49 (regenerant)
SH 97- 49 (Mother plant)
SH 97- 15
SH 97- 34-1
SH 97- 34-2

B. Variation for Esterase bands: from L to R
Test sample
SH 97- BS1
SH 97- BS2
Test sample
SH 97- 49 (Regenerant)
SH 97- 49 (Mother plant)
SH 97- 15
SH 97- 34-1
SH 97- 34-2

C. Variation for GOT bands: from L to R
SH 97- 34-2
SH 97- 34-1
SH 97- 15
SH 97- 49 (Mother plant)
SH 97- 49 (Regenerant)
Test sample
SH 97- BS2
SH 97- BS1
Test sample
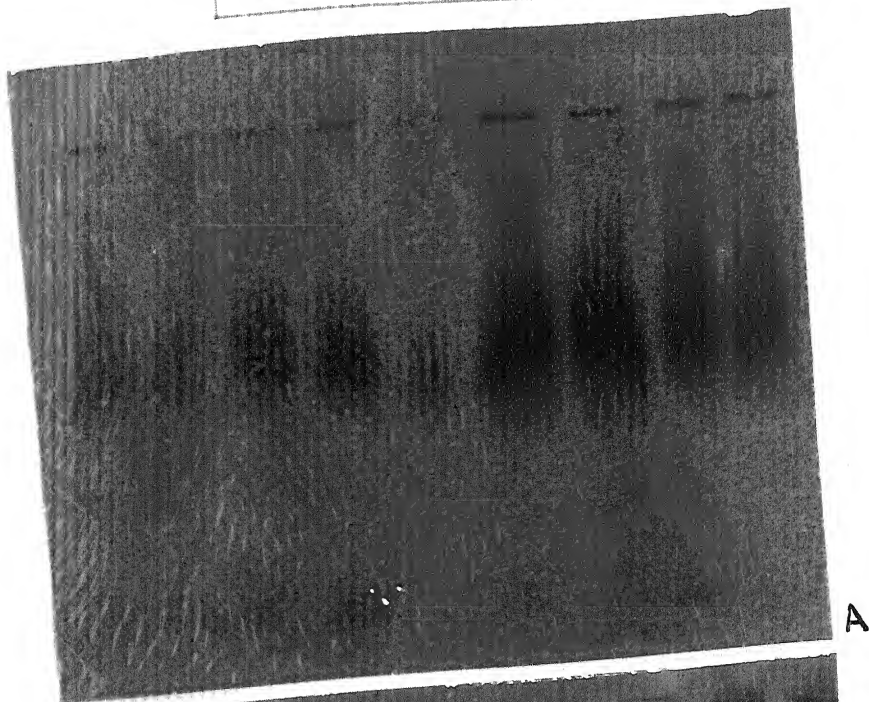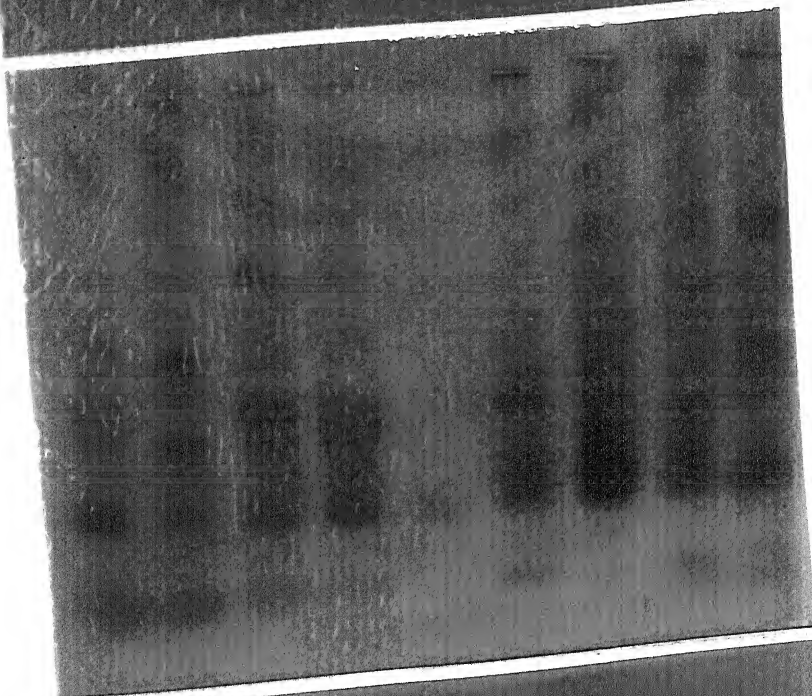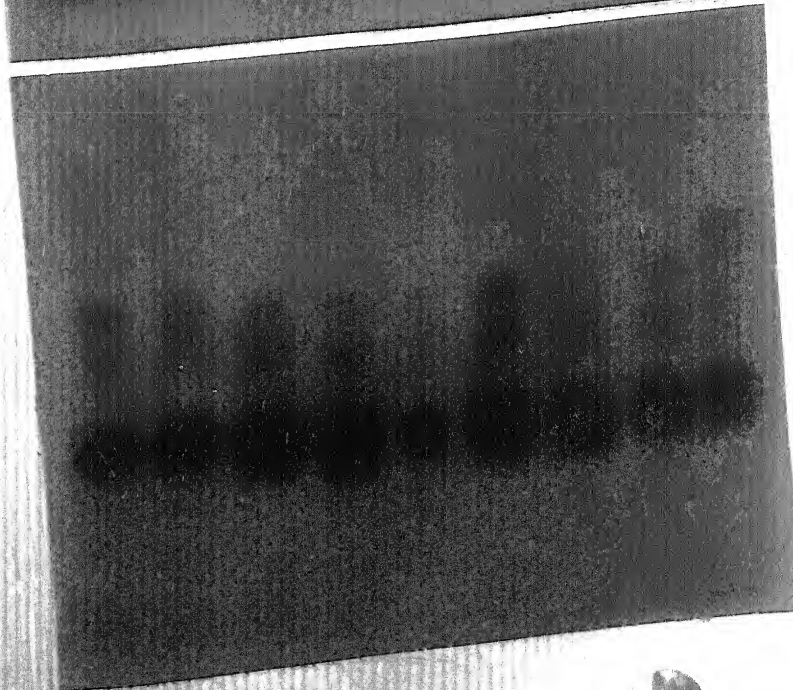
Plate : 11

Plate 12.   Isozyme variation in regenerant and the
mother plant of *T. resupinatum.* (SH 97-49)

    A. Variation for SOD bands: from L to R
       SH 97- 34-2
       SH 97- 34-1
       SH 97- 15
       SH 97- 49 (Mother plant)
       SH 97- 49 (Regenerant)
       Test sample
       SH 97- BS2
       SH 97- BS1
       Test sample

    B. Variation for Peroxidase bands: from L to R
       SH 97- 34-2
       SH 97- 34-1
       SH 97- 15
       SH 97- 49 (Mother plant)
       SH 97- 49 (Regenerant)
       Test sample
       SH 97- BS2
       SH 97- BS1
       Test sample

Plate : 12
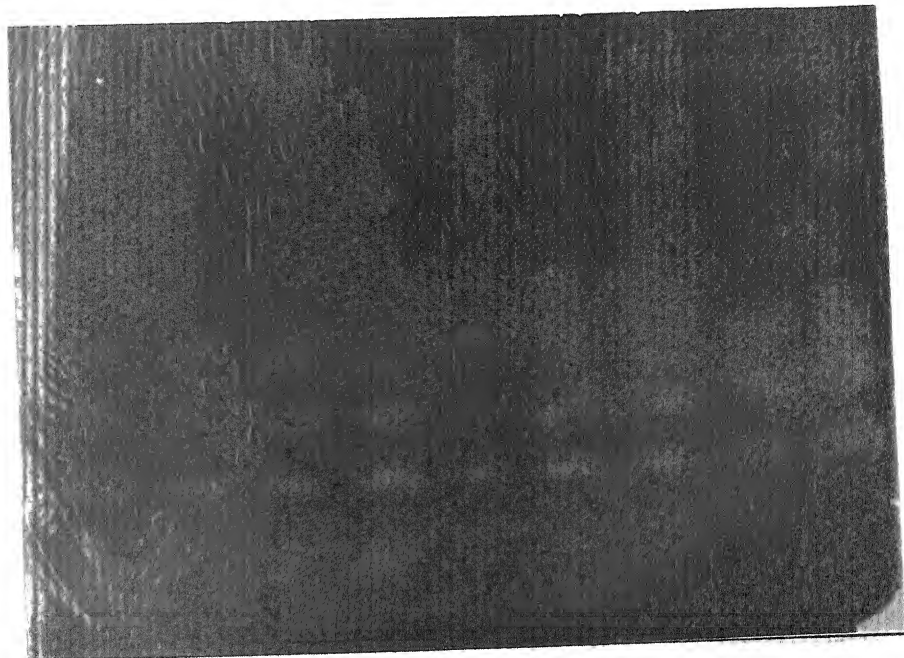
A
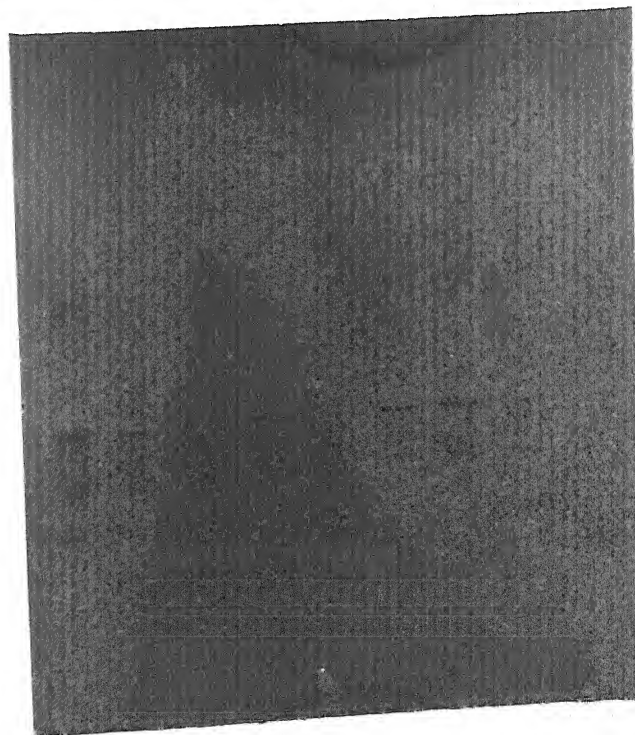
B

Plate 13. Isozyme variation among callus of different ages, mother plant and regenerant of *T. alexandrinum*.

A. Variation for Peroxidase in JHB 146: From L to R
>Test sample
>JHB 146 (Mother plant)
>40 days old calli
>60 days old calli
>90 days old calli
>300 days old calli
>Shooting regenerant
>Complete regenerant
>Flowering regenerant
> JHB 146 (Mother plant)
>Test sample

B. Variation for SOD in JHTB 1-90-P: From L to R
>Test sample
>JHTB 1-90-P (Mother plant)
>40 days old calli
>60 days old calli
>70 days old calli
>100 days old calli
>Shooting regenerant
> JHTB 1-90-P (Mother plant)
>Test sample

C. Variation for ACP in JHB 146:   From L to R
>Test sample
>JHB 146 (Mother plant)
>40 days old calli
>60 days old calli
>90 days old calli
>300 days old calli
>Shooting regenerant
>Complete regenerant
>Flowering regenerant
> JHB 146 (Mother plant)

D. Variation for ACP in JHTB 1-90-P: From L to R
>Test sample
>JHTB 1-90-P (Mother plant)
>40 days old calli
>60 days old calli
>70 days old calli
>100 days old calli
>Shooting regenerant
>JHTB 1-90-P (Mother plant)
>Test sample

Plate : 13
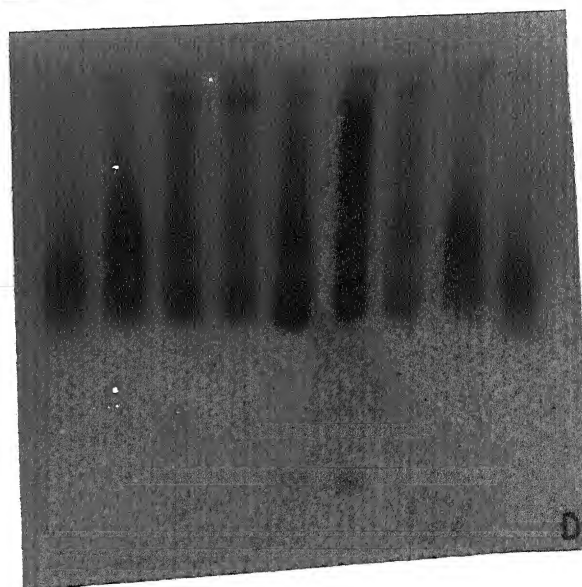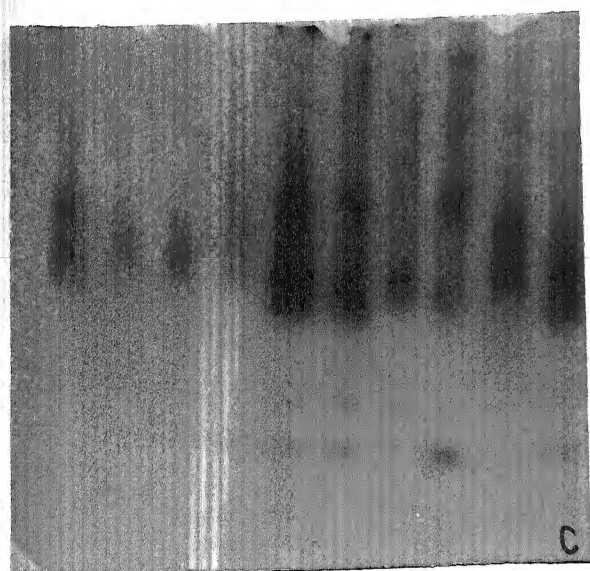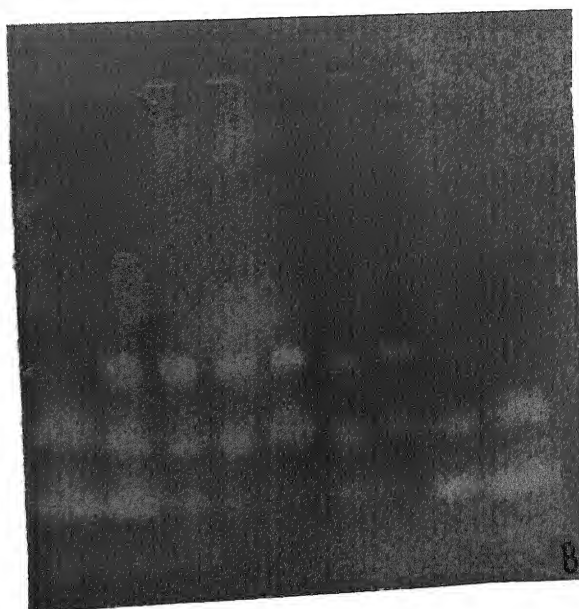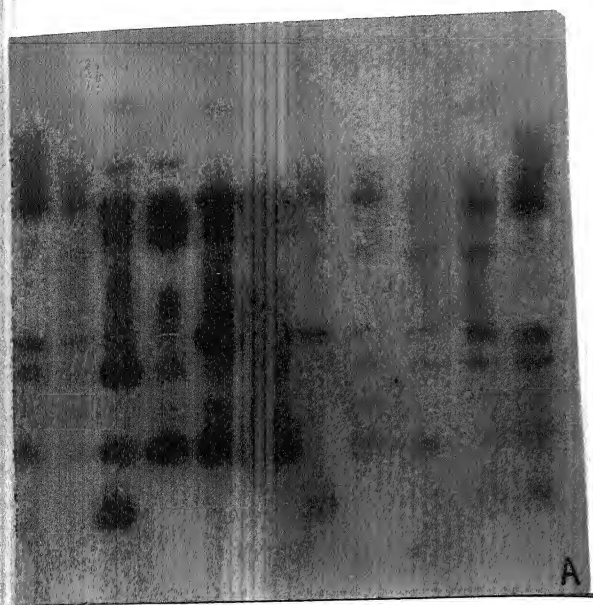
Plate 14. Somatic chromosomes of calli of different ages of diploid and tetraploid lines of *T. alexandrinum.*

A. A polyploid cell in callus developed from JHB 146

B. Early prophase showing two nucleolus and polyploid chromosomes in JHB 146

C. A tetraploid cell in the callus of JHB 146.

D. A tetraploid cell in the callus of JHTB 1-90-P

E. Diploid cell observed in calli of tetraploid JHTB 1-90-P

F. A polyploid cell at early prophase showing large number of chromosomes.

Plate : 14
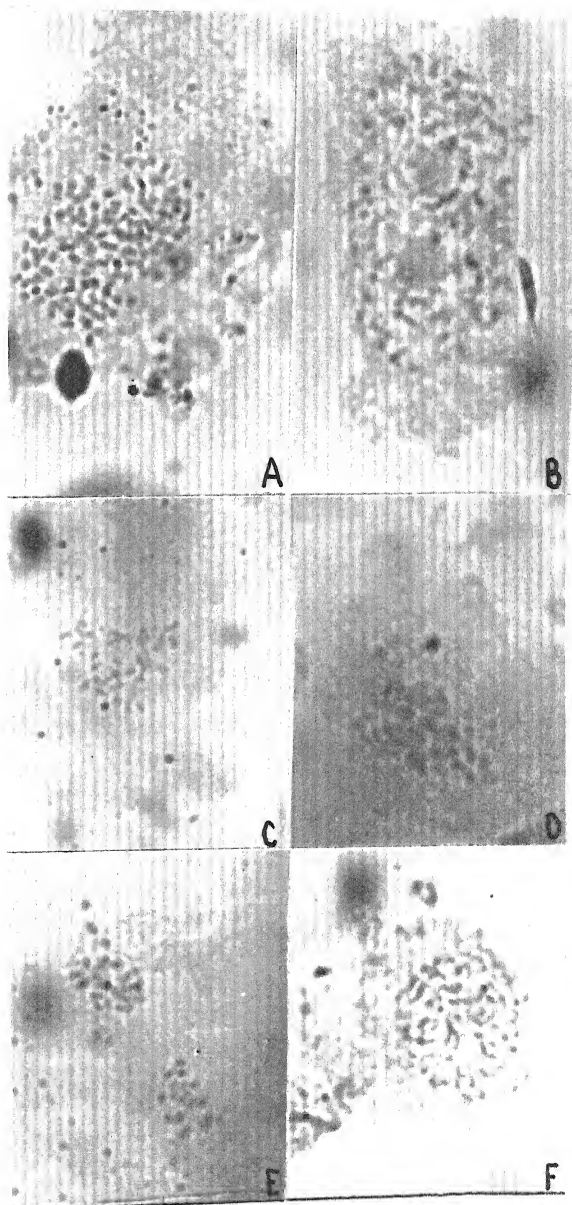
DISCUSSION

5. Discussion

The genus *Trifolium*, commonly called as clovers, comprises of several important forage and pasture species (Duke, 1981). These species are widely distributed in the temperate, sub-temperate and tropical parts of the world. Perennial species such as *T. repens* (white clover), *T. pratense* (red clover), *T. hybridum* (alsike clover) form the backbone of temperate livestock industry. In tropical countries such as India, *T. alexandrinum* (Berseem clover) and *T. resupinatum* (Persian clover or Shaftal) are very important cultivated forage crops.

Plant tissue culture technique has been developed and refined for many economically important plants during the last few decades. It exploits the theory of totipotency, which is probably a characteristic of all living plant cells. The technique has provided an efficient and powerful tool for generating, selecting and propagating novel and economically important plant genotypes.

To exploit the various biotechnological tools for genetic improvement of crops, it is imperative to have the suitable regeneration protocol. The *in vitro* regeneration of plants from callus is a pre-requisite to most of the biotechnological techniques (*viz.* somatic hybridization, genetic transformation etc) that have been proposed for the genetic manipulation of plants. Although totipotency is inherent in all living cells but its expression is not consistent and may vary with the physiological state of the explants, the genotype, the combination of growth hormones, inorganic and organic constituents of the culture media etc.

The present study was carried out to study the interaction of different factors such as genotype, media (growth regulator combinations), explants, age of explants etc. towards *in vitro* response.

Six species of *Trifolium* and 6 genotypes (3 diploid and 3 tetrapaloid) of *T. alexandrinum* were used which gave a wide range of genotypic variation for the study. A total of six explants were selected ranging from underground root to the leaf. The callus induction media combinations were selected after a few rounds of preliminary studies and literature survey. The selected media combinations include

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a wide range of different levels of auxins and cytokinins in various combination and ratio.

5.1. *In vitro* studies in different *Trifolium* species :

The experimental findings indicate differential response of various explants in different species. The response towards callus induction frequency, nature and growth rate of callus, its response towards organogenesis and differentiation varied widely in different explant-media-genotype combination. The outcome of experimental findings are discussed under various sub-headings.

5.1.1 Response of media-explant interaction in individual species :

5.1.1.1. *Trifolium resupinatum* (Shaftal or Persian clover) : It is an important forage of sub-tropical zone of the country. Frequently introduced in different parts of Europe and cultivated as fodder crop in north western sub-tropical zones.

Two media formulations were used to study the *in vitro* callus induction response in this species. The two media named as 'A' and 'A-1' differed in the growth hormone composition. A-1 medium comprised of L2 basal supplemented with BAP, whereas 'A' medium was provided with both auxin in the form of NAA and cytokinin in the form of BAP. Callogenic response was better in medium 'A' for all the explant source except collar. It indicate that media with both auxin and cytokinin content is better for callus induction. Regeneration potential was also good in the calli which were originally developed on A medium. The calli obtained from 'A-1' medium from any explant source did not show organogenesis and only callus proliferation was noticed and the calli turned brown after 15-20 days. Good organogenetic potential was noticed in the calli developed from cotyledon, hypocotyl and root in 'A' media where shoot formation was noticed. These shoots also produced roots when transferred to 'RL' medium. The study also points to the fact that although the calli was obtained in A-1 medium, it did not respond to organogenesis, which indicate the role of auxin in differentiation process. Thus the regeneration protocol was developed for this species.

5.1.1.2. *Trifolium subterraneum* L. (Subterranean clover) : This species is a slow growing runner annual to perennial clover, native of Southern Europe and

introduced as a pasture plant in countries including Australia, India and USA. It has good persistency and produces high quality forage.

In vitro interaction response in genotype IG 96-112 of this species was studied using 6 explants and two media formulations. The results indicated a media-explant dependent effect on callus induction and regeneration. The high hormone media 'D' showed better callogenic properties as compared to low hormone media 'A' for all the six explants studied. The callus induction response varied from 53.3% in cotyledon to 93.3% in root and leaf explants (Table 4.3). Very poor callus inducing response of 'A' media was observed for leaf, petiole, cotyledon, hypocotyl explants, while collar and root explants showed moderate response. Thus, for successful callus induction high amount of auxins and cytokinins are required.

Nature of callus formed was more dependent on explants than the media constitution. Calli obtained form leaf, cotyledon and collar were compact in nature, whereas those obtained from root were nodular in both the media. Similarly calli induced from hypocotyl and petiole were largely friable in nature. Chlorophyllous pigmentation could be induced only in a few calli obtained from leaf, hypocotyl and root in 'D' media and collar in 'A' media. Callus growth rate was also dependent on explant source, as leaf and cotyledon explants showed callus induction at a slow pace.

Organogenetic response of calli on subculturing to 'E' media was poor. In most of the cases only callus proliferation could be observed. The friable green calli obtained from 'D' media- hypocotyl combination showed induction and multiplication of shoots in 'E' media and root formation in 'RL' media. This combination thus, formed a successful protocol for regeneration of genotype IG 96-112 of *T. subterraneum.*

Embryogenic response of calli obtained from various combinations was poor. Green, globular embryo like structures were observed in some cases but these did not develop into plantlets.

MS media in which the inorganic salts are in higher concentration than in L2 media, have also been used by some workers to induce callus in this species but the success has not been encouraging. Graham (1968) could obtain only callus induction on modified MS media supplemented with Nicotinic acid, Kinetin and 2,4D but no morphogenetic response was observed.

The basal media used in the present study is L2 as described by Phillips and Collins (1984). Using the same basal media supplemented with the auxin Picloram (21μM) and maltose 4% as the sugar source calli were induced from shoot apices of cultivar Dalkeith (Heath *et al.*, 1993). Further organogenesis *via* shoot formation was achieved by using L2 basal media supplemented with 10.7 μM NAA and 4.4 μM BAP and 2.5% sucrose.

Parott and Collins (1982) also used L2 basal media for *T. subterraneum* and found rapid multiplication in shoot tip culture and prolific rooting on the standard rooting medium. However, callus did not grow well, exhibiting varying degrees of necrosis and they concluded that the L2 medium is suboptimal for this species. In our study also poor response towards callus induction was obtained in this media formulation. However, when the hormonal level was increased to 100 times in 'D' media, good callogenesis was observed.

5.1.1.3. *Trifolium repens* L. (White clover) : It is a perennial allotetraploid species and is the most important forage legume of the temperate pastures. The crop has attracted attention of scientists for developing varieties with desirable traits. Hence, in addition to conventional breeding methods, the biotechnological approach has also been attempted. Plant regeneration in *T. repens* has been reported by many workers. Pelletier and Pelletier (1971) regenerated plants from cotyledons cultured *in vitro*. Oswald *et al.*, (1977) obtained bud and plantlet regeneration with callus from seedlings as well as from cell suspension and protoplasts. Successful callus and cell suspension cultures were reported by Parrott and Collins (1982) where callus derived from 20% of the tested genotypes produced roots. Greshoff (1980) reported callus induction from seed and seedling explants on B5 or MS basal media supplemented with higher concentration of 2, 4-D.

In the present study, in genotype EC 400986 of *T. repens*, callogenic potential of root and collar explants was found to be very poor in both the low and high hormone media. Petiole explants showed good callus induction in both the media, whereas high hormone media 'D' was more effective for cotyledon and leaf explants. Calli obtained from leaf and cotyledon were compact and green. Friable calli were obtained in high frequency only from petiole explants. In all the combinations calli growth rate was slow. Response of various explants and media has been tried earlier also. White and Voisey (1994) conducted a series of experiments to screen the potential of roots, hypocotyls and cotyledons from seedlings to regenerate. Results indicated that cotyledon from 3 day old seedlings were most responsive.

As regards shoot and root induction, calli from petiole explants from both media showed induction of shoots in equal frequency. However, root induction could be obtained only in shoots derived from calli induced in 'D' media. Success rate was good for field transfer, as 40% plants could be transferred to field. Results obtained by White and Voisey (1994) indicate that the most prolific and rapid plant regeneration occurred on MS based media containing NAA and BAP, while other phytohormone combinations, 2, 4-D or picloram with kinetin 2-ip resulted in either extensive callus formation or distorted shoot development. They did not find much difference in frequency of shoot formation in eight cultivars of *T. repens*. Thus, our results are in confirmation of earlier results that NAA and BAP are good for regeneration in *T. repens* and its concentration during callusing from petiole has no bearing on shooting.

5.1.1.4. *Trifolium hybridum* L. (Alsike clover) : It is an erect perennial and valuable species of cultivated clovers, meadows and pastures indigenous to Europe.

The genotype (EC 401702) showed very good frequency of callus induction in all the combinations except for hypocotyl, and cotyledon explants in 'A' media. Callus induction was excellent in leaf, collar, petiole, root in both the media. Nature of the calli obtained was very much dependent on explant source as the leaf, cotyledon and collar showed compact calli whereas friable, green calli

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with better growth rate was found in hypocotyl and petiole explants which showed organogenesis after subculturing in shoot inducing media. Successful regeneration could be obtained in hypocotyl induced calli on both media. Petiole induced calli on media 'A' resulted in shoot formation and multiplication in 'E' media but rooting could not be induced. Leaf and collar although responded to callus formation in both the media but no differentiation could be observed on sub-culture, as most of the calli turned brown after sub-culturing.

Previous studies do not report regeneration protocol in this species. Callus growth was obtained from seedlings of Alsike clover cultured on SH medium containing 2.2 µM 2,4-D, 11µM CPA and 0.5 µM KIN, however, no morphogenetic development was reported (Schenk and Hildebrandt, 1972). Hence, this is the first successful regeneration of complete plantlet of this species.

5.1.1.5. *Trifolium apertum* : It is a diploid species domesticated in Russia and possesses close phenotypic resemblance with *T. alexandrinum*. This species has not been included in any tissue culture study earlier but due to its close affinity with *T. alexandrinum*, it was included in present study.

In the genotype EC 401712 of this species, an interesting observation was found that low hormone media 'A' failed to show callus induction in 5 out of 6 explants tried. Only in hypocotyl very low frequency of callus induction was observed and the calli were compact and green. Media 'D' showed good callogenesis in hypocotyl, cotyledon and petiole explants. In collar, leaf and root explants the frequency of callus induction was from nil to 14%. Friable green calli was observed in hypocotyl and petiole explants in 'D' media which also showed medium growth rate. Thus, this combination was found to be ideal for callus induction. The calli from petiole explant – 'D' media combination showed very good organogenetic and embryogenetic potential on sub-culture. 50% of sub-cultured tubes showed shoot induction and a large number of them also showed root induction in rooting media. Thus, the study was successful in developing a suitable protocol for *in vitro* regeneration and transfer of plantlets to field in this genotype.

5.1.1.6. *Trifolium glomeratum* : It is an annual species with good forage quality. The species is frequently found in dry places of Europe.

The *in vitro* study in genotype EC 401700 of *T. glomeratum* showed no response of root and collar explants to callus induction whereas in other combinations, media - explant interaction was observed to be good. Media 'A' was more effective for hypocotyl explant whereas cotyledon and petiole explants were more responsive to media 'D' so far as callus inducing efficiency is concerned. Friable, green calli could be obtained only in petiole –'D' media combination which also showed good organogenetic potential. The plantlets could be recovered and transferred to the field by successful root and shoot induction in these calli. The calli obtained from other combinations showed only callus proliferation and no organogenesis was observed.

5.1.2. Genotype –media interaction in *Trifolium* species :

The response of 5 different *Trifolium* species varied in two media. In general, low hormone media 'A' was less effective (23.86%) as compared to 'D' (54.38%) for callus induction (Table 5.1). Response of 'A' media was very good in *T. hybridum* (63.11%), while it was very poor in *T. apertum*. Furthermore, the calli induced on 'D' media showed good differentiation and most of the successful regeneration were obtained from calli induced on 'D' media.

5.1.3. Genotype-explant source interaction in *Trifolium* species :

Out of the six explants used in the present study, petiole responded best (61.93%) for callus induction. The response of root and collar was very poor for *T. repens*, *T. apertum* and *T. glomeratum* (Fig. 5.5, 5.6). Hypocotyl and cotyledon gave good friable calli in most of the cases. Very good callus induction was observed in leaf, petiole and collar explants of *T. hybridum*. Leaf explants performed very poor for callogenesis in *T. apertum* and *T. glomeratum*. The calli induced from hypocotyl showed regeneration potential and shoot induction was observed mostly in the calli induced from hypocotyl.

Table 5.1 : 2-way table giving explant source-media-genotype interaction for callus induction in different *Trifolium* species

Genotype - Media interaction (callus induction %)

	A	D	Mean
T. subterraneum	23.28	74.22	**48.75**
T. repens	18.33	43.22	**30.78**
T. hybridum	63.11	81.00	**72.06**
T. apertum	1.11	44.56	**22.83**
T. glomeratum	13.45	28.89	**21.17**
Mean	**23.86**	**54.38**	**39.12**

Genotype - Explant source interaction (callus induction %)

	Leaf	Petiole	Cotyledon	Hypocotyl	Collar	Root	Mean
T. subterraneum	52.50	41.00	32.33	36.67	58.34	71.67	**48.75**
T. repens	40.00	88.34	51.34	5.00	0.00	0.00	**30.78**
T. hybridum	100.00	93.33	25.00	48.34	100.00	65.67	**72.06**
T. apertum	7.00	48.34	26.67	50.00	0.00	5.00	**22.83**
T. glomeratum	5.00	38.67	48.33	35.00	0.00	0.00	**21.17**
Mean	**40.90**	**61.93**	**36.73**	**35.00**	**31.67**	**28.47**	**39.12**

Media - Explant source interaction (callus induction %)

	Leaf	Petiole	Cotyledon	Hypocotyl	Collar	Root	Mean
A	23.67	41.47	4.27	17.47	30.00	26.27	**23.86**
D	58.13	82.40	69.20	52.53	33.33	30.67	**54.38**
Mean	**40.90**	**61.93**	**36.73**	**35.00**	**31.67**	**28.47**	**39.12**

ANOVA table for callus induction in different *Trifolium* species

source	df	ss	ms	F
a	4	22015.705	5503.926	6.568**
b	1	13973.582	13973.582	16.674**
c	5	7153.249	1430.650	1.707
axb	4	3008.085	752.021	0.897
axc	20	27255.183	1362.759	1.626
bxc	5	6875.478	1375.096	1.641
axbxc	20	16760.784	838.039	
total	59			

a= species   b= media   c= explant source

**= signficant at 1% level

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Fig 5.1: Callusing response (%) of leaf explants on two media (A & D) in different *Trifolium* species
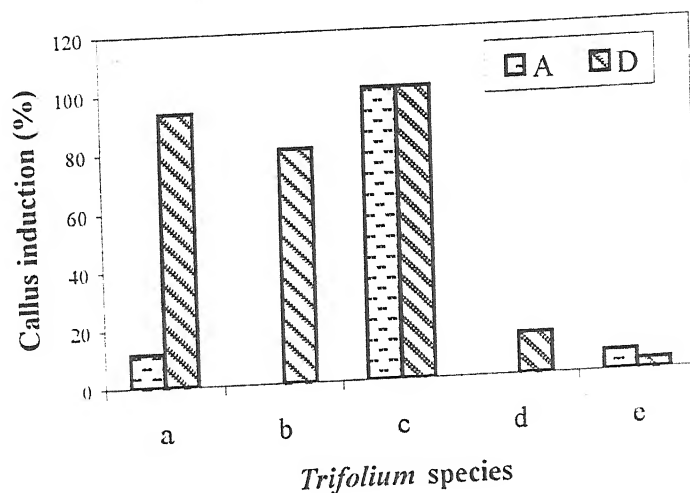
Fig 5.2: Callusing response (%) of petiole explants on two media (A & D) in different *Trifolium* species
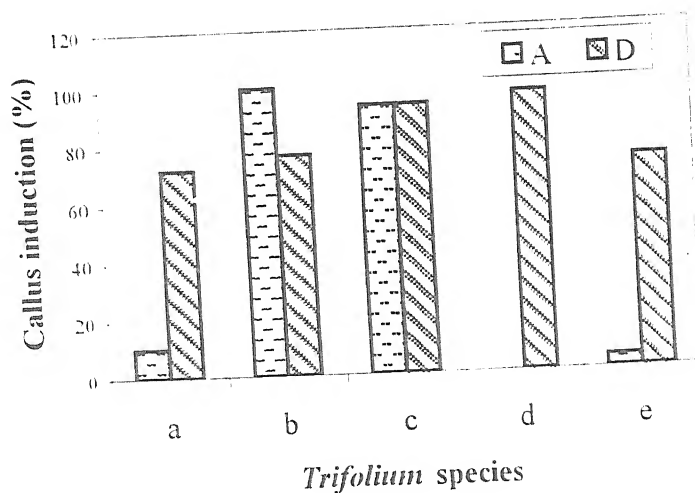
a = *T. subterraneum*   b = *T. repens*   c = *T. hybridum*
d = *T. apertum*   e = *T. glomeratum*

Fig 5.3: Callusing response (%) of hypocotyl explants on two media (A & D) in different *Trifolium* species
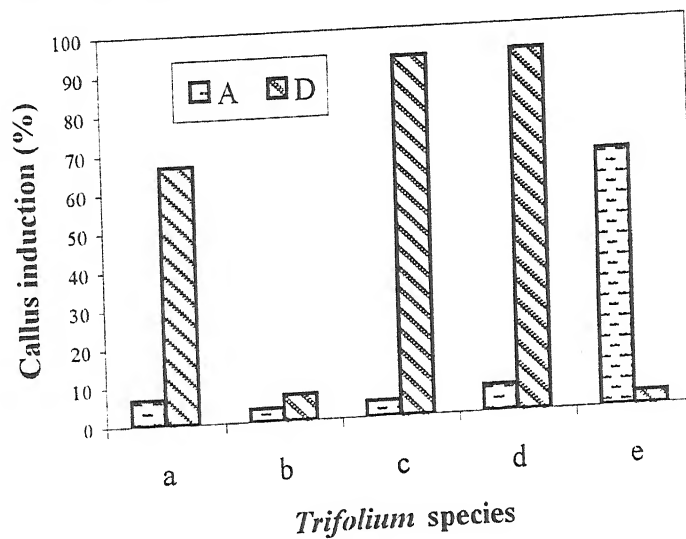
Fig 5.4: Callusing response (%) of cotyledon explants on two media (A & D) in different *Trifolium* species

a = *T. subterraneum*   b = *T. repens*   c = *T.hybridum*
d = *T. apertum*   e = *T. glomeratum*

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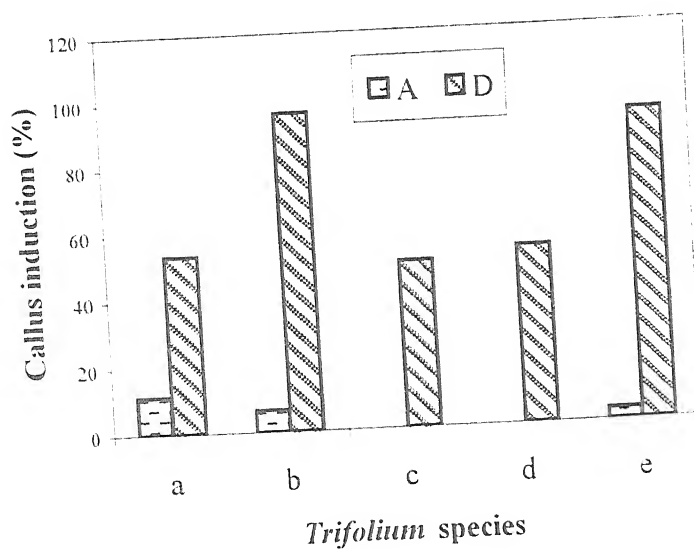
Fig 5.5: Callusing response (%) of collar explants on two media (A & D) in different *Trifolium* species
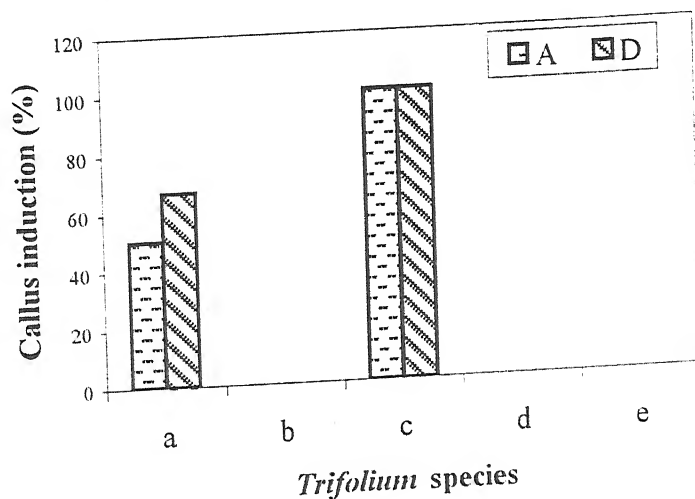
Fig 5.6: Callusing response (%) of root explants on two media (A & D) in different *Trifolium* species
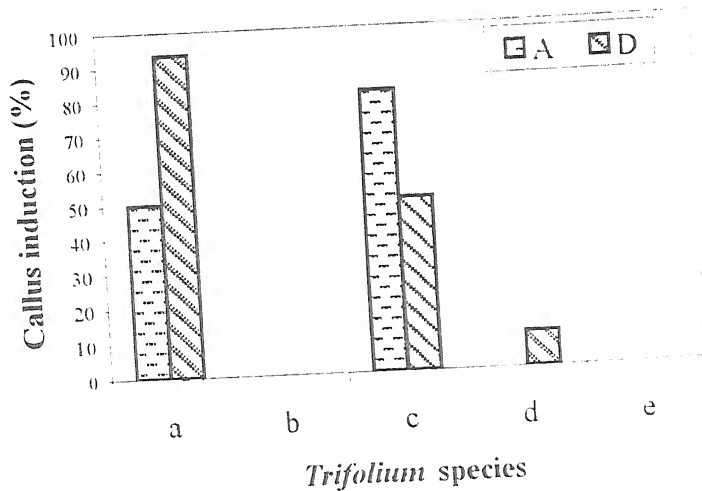
a = *T. subterraneum*   b = *T. repens*   c = *T. hybridum*

d = *T. apertum*   e = *T. glomeratum*

5.1.4. Media-explant source interaction in *Trifolium* species :

In all the combinations, petiole explant cultured on 'D' media showed best callus induction (82.4%) followed by cotyledon explants on 'D' media (Table 5.1). In cotyledon explants callus induction was observed to be good in 'D' media, whereas response of 'A' media was very poor (Fig.5.4). Maximum differentiation of calli was observed in petiole-'D' media and hypocotyl-'D' media combination in different species. 3- way ANOVA analysis showed highly significant effect of genotypes (five species) and media, whereas interactive effects were non-significant (Table 5.1).

5.1.5. Overall *in vitro* response in *Trifolium* species

In all these five species, an apparent genotype, media explant response was observed (Fig.5.1 to 5.6). The callus induction frequency, nature of calli and their growth rate was found to be dependent on different combinations. Some species such as *T. hybridum* showed better response towards callus induction (72%) followed by *T. subterraneum* (48.7%) when all the combination were considered (Table 5.1). High hormone media 'D' was found to be far superior (54.3%) than low hormone media 'A' (23.9%) for callus induction when all the observations were summarized (Table 5.1). Media 'D' was more effective in all the six explant media combinations. The response was very high in leaf, petiole, cotyledon and hypocotyl explants. The differential response of explants of different species to varying hormonal concentration can be attributed to the nature of explant tissue, its genetic potential for regeneration and hence, its nutritional requirement. It has been observed that nutritional requirements for optimal growth of a tissue *in vitro* vary with the species and even tissues from different parts of a plant may have different requirements for satisfactory growth.

In the earlier classical study, Skoog and Miller (1957) demonstrated that ratio of auxin and cytokinin play a significant role in organogenesis. They demonstrated that in Tobacco, high ratio of auxin to cytokinin favoured root formation, the reverse favoured shoot formation and an intermediate ratio promoted callus proliferation. In our study, ratio of auxin and cytokinin were tried

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in many combinations for callus induction, and regeneration. The results show media-explant interaction effect and it varied with the species.

Response of root and collar explants was generally very poor except in *T. subterraneum* and *T. hybridum*. This may be due to the fact that the former species is prostrate with profuse growth and the latter species is a perennial and more persistent, thus, have more active growing roots. In general, response of petiole explants was best among the six explants studied.

It was observed that only friable, green calli were responsive to organogenesis in shoot inducing media (media 'E'). Successful shoot induction was observed only in calli derived from following combinations :

- *T. resupinatum*   : Hypocotyl - 'A', Cotyledon- 'A', Root- 'A' media
- *T. subterraneum* : Hypocotyl - 'D' media
- *T. hybridum*   : Petiole -'D' media, Hypocotyl – 'D' and 'A' media
- *T. glomeratum*   : Petiole - 'D' media
- *T. apertum*   : Petiole -'D' media
- *T. repens*   : Petiole -'D' and 'A' media

The root inducing media 'RL' as developed by Phillips and Collins (1984) was found to be good for all the species. Satisfactory results were obtained in inducing good quality roots in almost half of the shoots. Therefore, in this study only one media was tried and more efforts were put towards good quality callus induction and shoot induction, which seemed to be a problem in these species as reported by previous authors.

Thus, in all the genotypes petiole and hypocotyl in 'D' media was found to be the best combinations for morphogenetic callus induction. The study also resulted in development of protocol for regeneration of plants in all six species studied.

5.2. *In vitro* studies in *Trifolium alexandrinum* L. :

It is the most important cultivated forage legume in tropical climate that has its origin in Mediterranean zone. In India it is cultivated as winter annual and is widely adapted for its high yielding quality fodder.

The present study included six genotypes (3 diploid, 3 tetraploid), four explant sources, two explant ages and 4 media combinations for callus induction and 4 media for shoot induction in different combinations. The findings are summarized here under in sub-headings of the factor which showed direct effect on callusing in regeneration response of different genotypes on different media.

5.2.1. Effect of seedling age of explants

The expression of totipotency may vary with the physiological state of the explants derived even from the same plant. Generally meristematic and embryonic cells yield calli of higher regeneration ability than do the mature and highly differentiated cells. Considering this fact 10 and 30 days old seedlings were used as explant source. It was considered that the different explants will vary in their developmental stage when excised from 10 and 30 days old seedlings. For example, the hypocotyl may be quite fast growing in 10 days old seedling but slow in the 30 days old seedling whereas the leaf explants will be slow in the 10 days old seedling but fast in the 30 days old seedling. Again the response of diploid and tetraploid seedling may vary.

Diploid genotypes: The callusing response of six genotypes under study was variable with respect to the age of the seedling, explant source and media. If we compare the individual response of diploid genotypes it revealed that there was no marked difference for young and old explants in the genotype FAO-1 for callus induction frequency (Table 5.2). Old collar explants showed better callus induction whereas other explants of young age were more responsive. Response of old explants was slightly better in high hormone media ('C' and 'D'). Significant interaction of explant age and explant source was observed (Table 5.2) in this genotype (FAO-1). Young explants of JHB 146 showed better (32.92%) response than old explants (26.25%), although it was not statistically significant. Old leaf

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Table 5.2 : 2-way table giving explant age - media - explant source interaction in genotype FAO-1 of _T. alexandrinum_

Explant age - Media interaction (callus induction %)

	A	B	C	D	Mean
Young	32.08	55.83	40.00	33.33	40.31
Old	24.17	50.84	53.34	40.83	42.29
Mean	28.13	53.33	46.67	37.08	41.30

Explant age - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
Young	10.42	65.83	45.00	40.00	40.31
Old	70.84	45.00	34.17	19.17	42.29
Mean	40.63	55.42	39.58	29.58	41.30

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	45.84	51.67	15.00	0.00	28.13
B	33.34	65.00	63.33	51.67	53.33
C	25.00	38.34	68.34	55.00	46.67
D	58.34	66.67	11.67	11.67	37.08
Mean	40.63	55.42	39.58	29.58	41.30

ANOVA table for callus induction in genotype FAO-1

source	df	ss	ms	F
a	1	31.383	31.383	0.048
b	3	2919.913	973.304	1.504
c	3	2719.542	906.514	1.401
axb	3	612.006	204.002	0.315
axc	3	9239.716	3079.905	4.760*
bxc	9	9613.489	1068.165	1.651
axbxc	9	5823.801	647.089	
total	31			

Factor a = Explant age   b = Media   c = Explant source

* = significant at 5% level

explants showed no callus induction in any media. This may be due to the fact that old leaves may have more permanent cells and less meristematic tissue. Old explants of collar and hypocotyl showed better response than younger ones, while the reverse was true for slow growing part such as petiole. Significant effect of explant source and explant age was observed in this genotype (Table 5.3).The third diploid genotype BL 142 showed better response of young explants for callus induction frequency than the older explants of collar, hypocotyl and leaf. Old petiole explants were more successful than younger ones. High hormone media 'C' and 'D' induced calli in higher number in young explants whereas in low hormone media ('A' and B) the callus induction response was similar in both the age groups. Significant effect of explant source was observed in this genotype (Table 5.4).

Tetraploid genotypes : It seems that the source of explants has a clear effect on the callogenic potential of two age groups as observed in tetraploid genotype 3-90 H. While the young meristematic zone derived explants such as collar and hypocotyl had a very high callus induction in old age, there was little difference in petiole and reverse was true for leaf. Leaf explants when fully mature showed no callogenic response while younger leaves showed good percentage of callus induction. High hormone media 'D' was more responsive in young explants whereas low hormone level showed better response for older explants. In general, older explants showed better response (48.1%) as compared to young explants (33.96%) (Table 5.5). Significant effect of explant source and interaction of explant age-explant source was observed.

The response of genotype 9-90N also showed marked difference for callogenesis with respect to age of the explant. The old explants showed poor response in all the media and explants source whereas the young explant were better responsive. There was a significant difference in explant age for callogenic potential with younger explants (42.08%) proving themselves better than older ones (10.83%) (Table 5.6). Young explants of the third tetraploid genotype 1-90 P were superior (46.79%) than the older explants (20.83%) for callus induction in all the four explant source (Table 5.7). Older explants showed very poor response at two extremes of hormonal level ('A' and 'D'), and good response in media 'B' and 'C' which has intermediate level of growth regulators. Leaf and petiole explants

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Table 5.3 : 2-way table giving explant age - media - explant source interaction in genotype JHB 146 of *T. alexandrinum*

Explant age - Media interaction (callus induction %)

	A	B	C	D	Mean
Young	43.33	29.17	29.17	30.00	**32.92**
Old	36.67	35.84	22.50	10.00	**26.25**
Mean	40.00	32.50	25.83	20.00	**29.58**

Explant age - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
Young	28.33	40.84	34.17	28.33	**32.92**
Old	36.67	55.00	13.33	0.00	**26.25**
Mean	32.50	47.92	23.75	14.17	**29.58**

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	80.00	38.34	20.00	21.67	**40.00**
B	21.67	63.34	33.34	11.67	**32.50**
C	21.67	46.67	21.67	13.34	**25.83**
D	6.67	43.34	20.00	10.00	**20.00**
Mean	32.50	47.92	23.75	14.17	**29.58**

ANOVA table for callus induction in genotype JHB 146

	df	ss	ms	F
a	1	355.578	355.578	1.803
b	3	1783.442	594.481	3.014
c	3	4930.903	1643.634	8.333**
axb	3	711.156	237.052	1.202
axc	3	2658.317	886.106	4.493*
bxc	9	5647.270	627.474	3.181
axbxc	9	1775.117	197.235	
total	31			

Factor a = Explant age   b = Media   c = Explant source

* = significant at 5% level   ** = significant at 1% level

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Table 5.4 : 2-way table giving explant age - media - explant source interaction in genotype BL 142 of *T. alexandrinum*

Explant age - Media interaction (callus induction %)

	A	B	C	D	Mean
Young	38.33	40.83	45.84	33.34	39.58
Old	38.33	37.50	28.33	4.17	27.08
Mean	38.33	39.17	37.08	18.75	33.33

Explant age - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
Young	27.50	66.67	40.00	24.17	39.58
Old	0.00	36.67	63.33	8.33	27.08
Mean	13.75	51.67	51.67	16.25	33.33

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	11.67	68.34	51.67	21.67	38.33
B	30.00	55.00	61.67	10.00	39.17
C	13.34	41.67	78.34	15.00	37.08
D	0.00	41.67	15.00	18.34	18.75
Mean	13.75	51.67	51.67	16.25	33.33

ANOVA table for callus induction in genotype BL 142

source	df	ss	ms	F
a	1	1250.125	1250.125	2.132
b	3	2285.739	761.913	1.299
c	3	10780.678	3593.559	6.128*
axb	3	1086.247	362.082	0.617
axc	3	3652.681	1217.560	2.076
bxc	9	4067.183	451.909	0.771
axbxc	9	5277.947	586.439	
total	31			

Factor a = Explant age   b = Media   c = Explant source

* = significant at 5% level

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Table 5.5 : 2-way table giving explant age - media - explant source interaction in genotype 3-90 H of *T. alexandrinum*

Explant age - Media interaction   (callus induction %)

	A	B	C	D	Mean
Young	26.67	36.67	39.17	33.34	33.96
Old	62.50	54.17	55.84	20.00	48.13
Mean	44.58	45.42	47.50	26.67	41.04

Explant age - Explant source interaction   (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
Young	5.83	39.17	49.17	41.67	33.96
Old	62.50	71.67	58.34	0.00	48.13
Mean	34.17	55.42	53.75	20.83	41.04

Media - Explant source interaction   (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	25.00	68.34	75.00	10.00	44.58
B	50.00	43.34	58.34	30.00	45.42
C	61.67	50.00	58.34	20.00	47.50
D	0.00	60.00	23.34	23.34	26.67
Mean	34.17	55.42	53.75	20.83	41.04

ANOVA table for callus induction in genotype 3-90 H

source	df	ss	ms	F
a	1	1605.603	1605.603	3.598
b	3	2240.168	746.723	1.673
c	3	6590.635	2196.878	4.922*
axb	3	2486.256	828.752	1.857
axc	3	10569.756	3523.252	7.894**
bxc	9	6256.751	695.195	1.558
axbxc	9	4016.675	446.297	
total	31			

Factor **a** = Explant age   **b** = Media   **c** = Explant source

* = significant at 5% level   ** = significant at 1% level

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Table 5.6 : 2-way table giving explant age - media - explant source interaction in genotype 9-90 N of *T. alexandrinum*

Explant age - Media interaction (callus induction %)

	A	B	C	D	Mean
Young	31.67	52.50	30.83	53.34	42.08
Old	12.50	4.17	22.50	4.17	10.83
Mean	22.08	28.33	26.67	28.75	26.46

Explant age - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
Young	19.17	57.50	41.67	50.00	42.08
Old	12.50	12.50	18.34	0.00	10.83
Mean	15.83	35.00	30.00	25.00	26.46

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	25.00	25.00	21.67	16.67	22.08
B	11.67	43.34	20.00	38.34	28.33
C	6.67	38.33	40.00	21.67	26.67
D	20.00	33.34	38.34	23.34	28.75
Mean	15.83	35.00	30.00	25.00	26.46

ANOVA table for callus induction in genotype 9-90 N

source	df	ss	ms	F
a	1	7811.875	7811.875	21.207**
b	3	223.722	74.574	0.202
c	3	1604.308	534.769	1.452
axb	3	2568.444	856.148	2.324
axc	3	2415.547	805.182	2.186
bxc	9	1748.856	194.317	0.528
axbxc	9	3315.256	368.362	
total	31			

Factor a = Explant age   b = Media   c = Explant source

** = significant at 1% level

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showed better response in older explants than the collar and hypocotyl in this genotype. Significant effect of explant age and explant source was observed.

Thus, the age of various explant was found to be important factor affecting the callusing response in different media. This fact has also been observed by earlier workers. For example, George and Sharrington, (1984) reported that the explants of young tissues, generally form callus which undergo cell division more rapidly than older tissues. Clog *et al.* (1990) have also reported that, in V*itis*, the age of explant is very important for development of organogenic calli and the younger leaves were most efficient. Physiological maturity and segmental age of the explant plays an vital role in the callogenic response of the explant as observed in *Indigofera* by Ayyappan and Kumar (1989) wherein response of seedling explants was found to be better than leaf cultures.

5.2.2. Genotypic response

In any improvement programme involving biotechnological aspects it is always better to standardize the protocol for regeneration of the plantlet from the target species or the variety because the totipotentiality may not be consistently expressed by all cultivars of a species or all species of a genus. Mohapatra and Gresshoff (1982) screened a number of ecotypes of *T. repens* and were able to select genotypes capable of differentiating shoots from calli raised from petiole and root segments. Bhojwani *et al.* (1984) have also stressed on selection of genotypes and recommended screening of a large number of plants within a cultivar of outbreeding species to achieve plant regeneration from tissue culture. In an out-breeding species like *T. alexandrinum*, strains are highly heterogeneous population. Hence, in the present study six genotypes represented by two ploidy levels were selected.

Table 5.7 : 2-way table giving explant age - media - explant source interaction in genotype 1-90 P of *T. alexandrinum*					

Explant age - Media interaction (callus induction %)

	A	B	C	D	Mean
Young	61.33	32.50	59.17	34.17	46.79
Old	3.33	37.50	42.50	0.00	20.83
Mean	32.33	35.00	50.83	17.08	33.81

Explant age - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
Young	6.33	69.17	54.17	57.50	46.79
Old	3.33	0.00	37.50	42.50	20.83
Mean	4.83	34.50	45.83	50.00	33.78

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	19.33	45.00	40.00	25.00	32.33
B	0.00	25.00	40.00	75.00	35.00
C	0.00	48.34	85.00	70.00	50.83
D	0.00	20.00	18.34	30.00	17.08
Mean	4.83	34.58	45.83	50.00	33.81

ANOVA table for callus induction in genotype 1-90 P

source	df	ss	ms	F
a	1	5390.854	5390.854	10.113*
b	3	4585.397	1528.466	2.867
c	3	9975.847	3325.282	6.238*
axb	3	4277.594	1425.865	2.675
axc	3	5201.044	1733.681	3.252
bxc	9	5995.818	666.202	1.250
axbxc	9	4797.398	533.044	
total	31			

Factor **a** = Explant age **b** = Media **c** = Explant source

* = significant at 5% level

<u>Diploid genotypes :</u> Three diploid genotypes selected for this study were from different sources. Genotype FAO-1 is an exotic germplasm , JHB 146 is a high yielding variety developed at IGFRI, Jhansi and BL–142 is an advanced breeding line developed at PAU, Ludhiana. All the three genotypes belonged to Mescavi group.

The young seedling explants of all the diploid lines showed no difference for response to different media combination. The callogenic response was more or less similar in the three genotypes with JHB 146 showing a little poor response. Media 'B' was slightly better than other three media. Non- significant difference was observed for callus induction frequency in different media and genotypes, whereas, explant source showed significant difference in young explants (Table 5.8).

Similarly, no significant difference was observed in efficacy of different media combinations for callus induction from explants taken from the old seedlings whereas, explant source showed significant variation (Table 5.9). In this case too the intermediate hormone level media ('B' and 'C') were more effective than the media at two extremes of low and high hormonal concentrations 'A' and 'D'. Genotypes FAO-1 and BL 142 were equally effective whereas response of JHB 146 was little low.

<u>Tetraploid genotypes</u> : The explants derived from young seedling of three tetraploid genotypes were compared for callusing response in four media combination. Out of the three genotypes, 1-90p showed best response for callus induction followed by 9-90 N and 3-90 H. But, marked difference was observed in the response of three tetraploid genotypes for callus induction when explants were collected from 30 days old seedling. The callus induction ranged from 10.8% in 9-90 N to 48.1% in 3-90 H (Table 5.11). Intermediate hormone level media B and C were more effective. Very poor response was seen in high hormonal media for callus induction in tetraploid genotypes. Significant effect of genotypic variation was observed for callus induction (Table 5.11).

As observed in diploid genotypes very good response for callogenesis was observed for hypocotyl explant both in old and young seedling. The old petiole

Table 5.8 : 2-way table giving explants-media-genotypes interaction for callus induction in diploid *T. alexandrinum* (young seedling)

Genotype - media interaction (callus induction %)

Genotype	A	B	C	D	Mean
FAO--1	32.08	55.83	40.00	33.33	40.31
JHB 146	43.33	29.17	29.17	30.00	32.92
BL 142	38.33	40.83	45.84	33.34	39.59
Mean	37.92	41.94	38.33	32.23	37.60

Genotype - explant interaction (callus induction %)

Genotype	Collar	Hypocotyl	Petiole	Leaf	Mean
FAO--1	10.42	65.83	45.00	40.00	40.31
JHB 146	28.33	40.84	34.17	28.33	32.92
BL 142	27.50	66.68	40.00	24.17	39.59
Mean	22.08	57.78	39.72	30.83	37.60

Media - explant interaction (callus induction %)

Media	Collar	Hypocotyl	Petiole	Leaf	Mean
A	37.22	62.22	34.44	17.78	37.92
B	25.56	64.43	41.11	36.67	41.94
C	15.56	43.33	52.22	42.22	38.33
D	10.00	61.12	31.11	26.67	32.23
Mean	22.08	57.78	39.72	30.83	37.60

ANOVA table for callus induction in young explants of diploid genotypes

source	df	ss	ms	F
a	2	531.49	265.75	0.42
b	3	580.55	193.52	0.31
c	3	8378.66	2792.89	4.42*
axb	6	1753.26	292.21	0.46
axc	6	2784.25	464.04	0.73
bxc	9	3396.01	377.33	0.60
axbxc	18	11368.22	631.57	
total	47			

Factor a= Genotype   b= media   c=explant

* = significant at 5% level

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Table 5.9 : 2-way table giving explants-media-genotypes interaction for callus induction in diploid T. alexandrinum (old seedling)

Genotype - Media interaction (callus induction %)

	A	B	C	D	Mean
FAO--1	24.17	48.34	53.34	40.83	41.67
JHB 146	36.67	35.84	22.50	10.00	26.25
BL 142	38.33	40.83	45.84	33.34	39.59
Mean	33.06	41.67	40.56	28.06	35.83

Genotype - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
FAO--1	70.84	45.00	34.17	16.67	41.67
JHB 146	36.67	55.00	13.33	0.00	26.25
BL 142	27.50	66.68	40.00	24.17	39.59
Mean	45.00	55.56	29.17	13.61	35.83

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	62.22	44.44	22.22	3.33	33.06
B	51.11	61.11	38.89	15.56	41.67
C	33.33	60.00	45.56	23.33	40.56
D	33.33	56.68	10.00	12.22	28.06
Mean	45.00	55.56	29.17	13.61	35.83

ANOVA table for callus induction in old explants of diploid genotypes

source	df	ss	ms	F
a	2	2239.319	1119.660	2.090
b	3	1494.056	498.019	0.930
c	3	12136.387	4045.462	7.553**
axb	6	2694.864	449.144	0.839
axc	6	5671.199	945.200	1.765
bxc	9	3804.441	422.716	0.789
axbxc	18	9640.974	535.610	
total	47			

Factor a = Explant age   b = Media   c = Explant source

** = significant at 1% level

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Table 5.10 : 2-way table giving explants-media-genotypes interaction for callus induction in tetraploid *T. alexandrinum* (young seedling)

Genotype - media interaction   (callus induction %)

Genotype	A	B	C	D	
3-90 H	26.67	36.67	39.17	33.34	33.96
9-90 N	31.67	52.50	30.83	53.34	42.08
1-90 P	61.33	32.50	59.18	34.17	46.79
Mean	39.89	40.56	43.06	40.28	40.95

Genotype - explant interaction   (callus induction %)

axc	Collar	Hypocotyl	Petiole	Leaf	
3-90 H	5.83	39.17	49.17	41.67	33.96
9-90 N	19.17	57.50	41.67	50.00	42.08
1-90 P	6.33	69.18	54.17	57.50	46.79
Mean	10.44	55.28	48.33	49.72	40.95

Media - explant interaction   (callus induction %)

BXC	Collar	Hypocotyl	Petiole	Leaf	
A	8.44	58.89	57.78	34.44	39.89
B	7.78	52.22	40.00	62.22	40.56
C	12.22	61.12	47.78	51.11	43.06
D	13.33	48.89	47.78	51.11	40.28
Mean	10.44	55.28	48.33	49.72	40.95

ANOVA table for callus induction in young explants of tetraploid genotypes

source	df	ss	ms	F
a	2	1348.952	674.476	5.428*
b	3	74.046	24.682	0.199
c	3	15209.997	5069.999	40.798**
axb	6	5072.690	845.448	6.803
axc	6	1757.000	292.833	2.356
bxc	9	1944.214	216.024	1.738
axbxc	18	2236.847	124.269	
total	47			

a= Genotype   b= media   c=explant

* = significant at 5% level   ** = significant at 1% level

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Table 5.11 : 2-way table giving explants-media-genotypes interaction for callus induction in tetraploid *T. alexandrinum* (old seedling)

Genotype - Media interaction (callus induction %)

	A	B	C	D	Mean
3-90 H	62.50	54.17	55.84	20.00	48.13
9-90 N	12.50	4.17	22.50	4.17	10.83
1-90 P	3.33	37.50	42.50	0.00	20.83
Mean	26.11	31.95	40.28	8.06	26.60

Genotype - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
3-90 H	62.50	71.67	58.34	0.00	48.13
9-90 N	12.50	12.50	18.34	0.00	10.83
1-90 P	3.33	0.00	37.50	42.50	20.83
Mean	26.11	28.06	38.06	14.17	26.60

Media - Explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	37.78	33.33	33.33	0.00	26.11
B	33.33	22.22	38.89	33.33	31.95
C	33.33	30.00	74.45	23.33	40.28
D	0.00	26.67	5.56	0.00	8.06
Mean	26.11	28.06	38.06	14.17	26.60

ANOVA table for callus induction in old explants of tetraploid genotypes

source	df	ss	ms	F
a	2	11922.944	5961.472	6.736**
b	3	6717.494	2239.165	2.530
c	3	3458.420	1152.807	1.303
axb	6	4518.093	753.016	0.851
axc	6	15933.062	2655.510	3.001*
bxc	9	6020.626	668.958	0.756
axbxc	18	15929.976	884.999	
total	47			

Factor a = Explant age   b = Media   c = Explant source

* = significant at 5% level   ** = significant at 1% level

Fig 5.7: Genotype-media interaction in *Trifolium alexandrinum* (Young seedling)
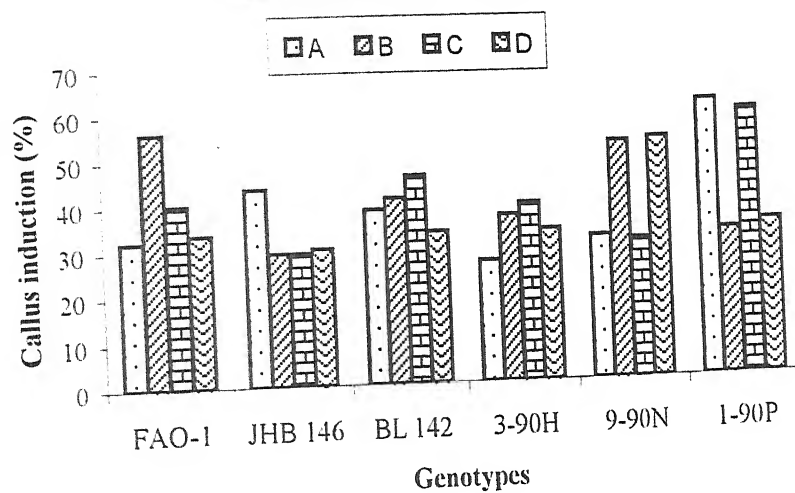

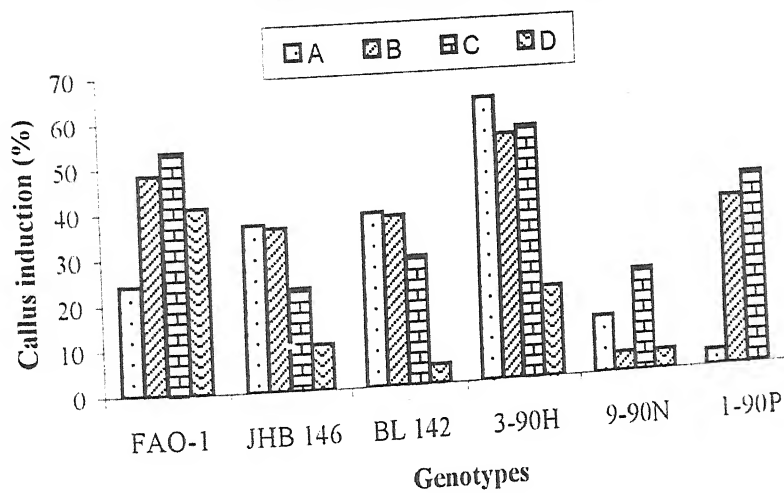Fig 5.8: Genotype-media interaction in *Trifolium alexandrinum* (Old seedling)

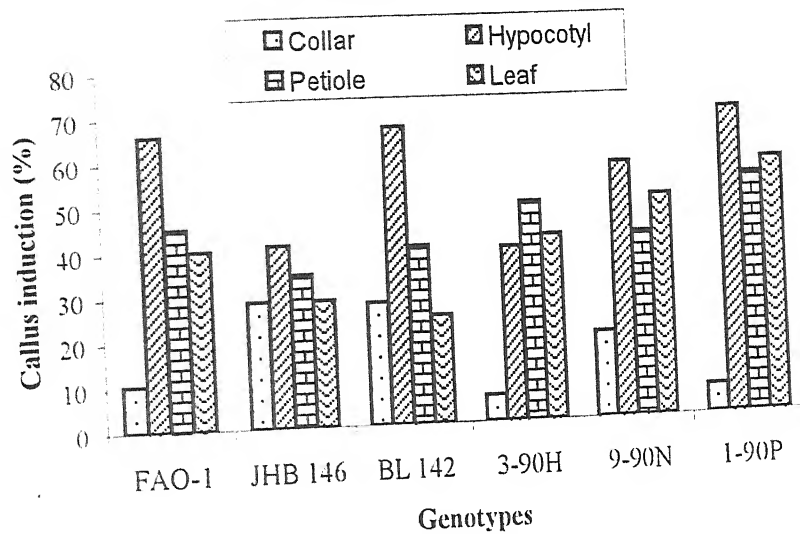Fig 5.9: Genotype-explant interaction in *Trifolium alexandrinum* (Young seedling)

Fig 5.10: Genotype-explant interaction in *Trifolium alexandrinum* (Old seedling)
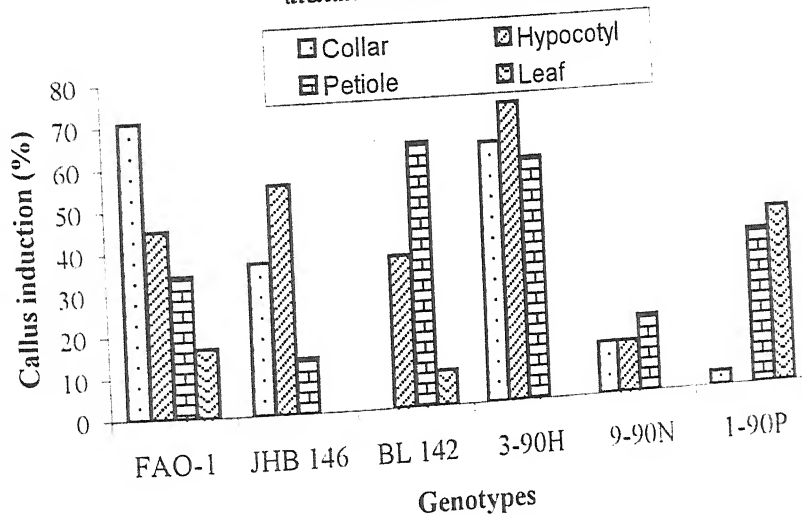
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Fig 5.11. Media - explant interaction in *Trifolium alexandrinum* (Young seedling)
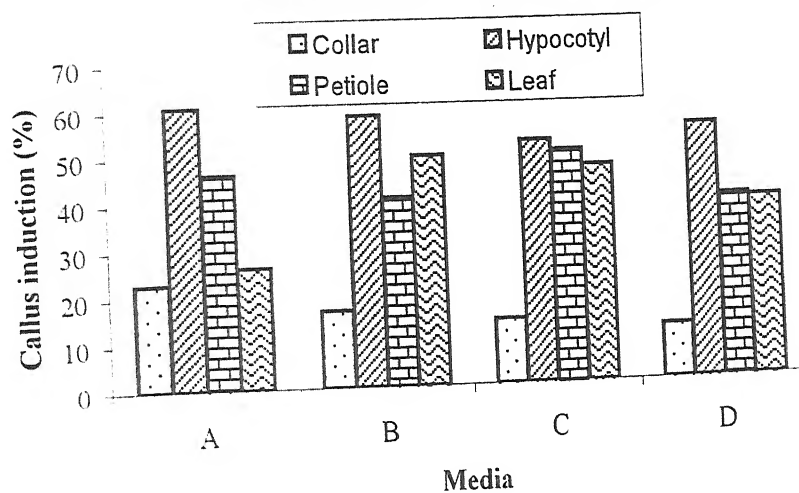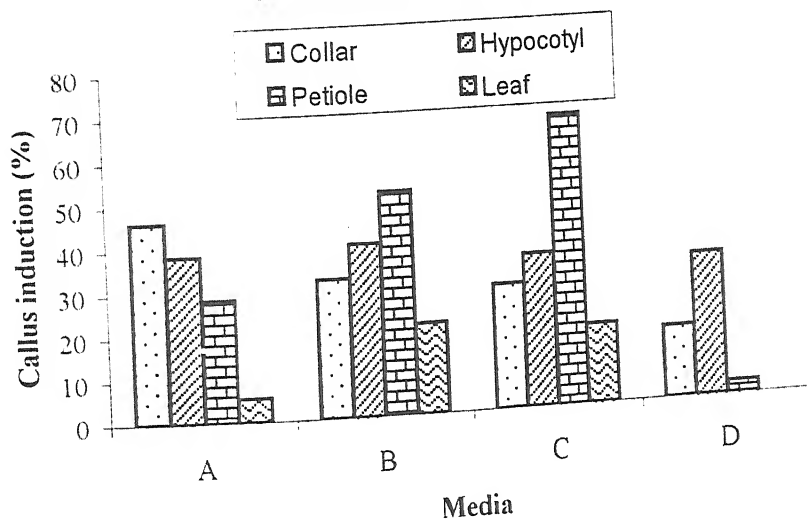

Fig 5.12: Media - explant interaction in *Trifolium alexandrinum* (Old seedling)

explants also responded well to different media. The young collar and old leaf explants showed very poor response.

Response of different young explants of six genotypes of *T. alexandrinum* to four media combination was also compared and it was observed that hypocotyl was best responsive followed by petiole and leaf. Least response was observed in collar derived explants (Table 5.11, 5.12).

Very high significant effect of genotype, media, explant source were observed in old seedling stage experiments when the data of all six genotypes were considered (Table 5.13), which shows that the callogenic property of old explants varied widely in different genotypes. Interactive effect of genotype and explant source were also significant (Table 5.13).

5.2.3. Media formulation response :

Four media formulations ('A', 'B',' C', 'D') with varying level of hormones were used in the present study. Ratio of auxin to cytokinin also varied in these media. In media 'A' auxin : cytokinin ratio was kept as 0.5:1, whereas, in other 3 media it was 5:1 (Table 3.2). The concentration of hormones also varied widely. Media 'A' was having lowest level of hormone which gradually increased in 'B' and 'C'. Media 'D' had very high level of NAA (5.0 mg/L) and BAP (1.0 mg/L).

The media response to callogenesis and subsequent regeneration was found to be dependent on its interaction with genotype and explants (Fig. 5.7, 5.8, 5.11, 5.12). In the young seedling all 4 media behaved almost equally when the data for all 6 genotypes were pooled (Table 5.12). Whereas, in old explants, media 'D' performed very poorly (13.2%) as compared to media 'C' (37.5%) and media 'B' (36.25%) (Table 5.13). Very poor response of tetraploid genotype 1-90P was observed in 'A' and 'D' media, whereas it was very good in young explant of same genotype.

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5.2.4. Explants source response:

In the present study four explant source i.e. leaf, petiole, collar and hypocotyl were used. As these explants were excised from six genotypes at two different growth stages and cultured on four media combinations supplemented with varying degree of hormones, the response of different explants was compared in different genotype, media and seedling stage combination (Fig. 5.9, 5.10).

In diploid genotypes, young hypocotyl explants were found to be far better (57.78%) than other explants source for callus induction capability. Collar showed poor response (22.08%) whereas petiole and leaf showed moderate response with petiole (39.7%) performing better than leaf (30.8%) (Table 5.8). In tetraploid genotypes also hypocotyl was best among the four explants tried. Leaf and petiole showed equal callogenic capability whereas response of collar derived explants was poor (10.4%) (Table 5.10).

As regards the overall response of different media to the four young explants, no marked difference was observed i.e. high or low hormone concentration were equally effective to the explants (Table 5.12) except that response of leaf was very poor..

The explants excised from 30 days old seedling showed differential response of different explants to callus induction. At old seedling stage, among diploid genotypes, hypocotyl, petiole, collar explants showed better response in comparison to leaf where callus inducing response was poor (Table 5.13). Highly significant effect of explant source was observed in both young and old seedling explants (Table 5.12, 5.13).

The various calli from the same callus mass behaved differently on sub-culture to the same media. While some calli did not show any response, other responded either by proliferation or by differentiation leading to shoot induction. It indicates that the calli at the time of sub-culture were not homogeneous.

The inherent heterogeneity in the callus culture has been attributed to the culture conditions. As there is a unidirectional supply of nutrients (from the medium below) and gases and light (predominantly from above), chemical and

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Table 5.12 : 2-way table giving explants-media-genotypes interaction for callus induction in different *T. alexandrinum* genotypes (young seedling)

Genotype - Media interaction (callus induction %)

	A	B	C	D	
FAO-1	32.08	55.83	40.00	33.33	40.31
JHB 146	43.33	29.17	29.17	30.00	32.92
BL 142	38.33	40.83	45.84	33.34	39.59
3-90 H	26.67	36.67	39.17	33.34	33.96
9-90 N	31.67	52.50	30.83	53.34	42.08
1-90 P	61.33	32.50	59.18	34.17	46.79
Mean	38.90	41.25	40.70	36.25	39.27

Genotype - explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	
FAO-1	10.42	65.83	45.00	40.00	40.31
JHB 146	28.33	40.84	34.17	28.33	32.92
BL 142	27.50	66.68	40.00	24.17	39.59
3-90 H	5.83	39.17	49.17	41.67	33.96
9-90 N	19.17	57.50	41.67	50.00	42.08
1-90 P	6.33	69.18	54.17	57.50	46.79
Mean	16.26	56.53	44.03	40.28	39.27

Media - explant source interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	22.83	60.56	46.11	26.11	38.90
B	16.67	58.33	40.56	49.45	41.25
C	13.89	52.23	50.00	46.67	40.70
D	11.67	55.01	39.45	38.89	36.25
Mean	16.26	56.53	44.03	40.28	39.27

ANOVA table for young explants of different *T. alexandrinum* genotypes

source	df	ss	ms	F
a	5	2148.314	429.663	1.190
b	3	364.569	121.523	0.337
c	3	20420.389	6806.796	18.856**
axb	15	7115.983	474.399	1.314
axc	15	7709.512	513.967	1.424
bxc	9	2701.046	300.116	0.831
axbxc	45	16244.237	360.983	
total	95			

a= Genotype		b= media	c=explant

** = significant at 1% level

Table 5.13 : 2-way table giving explants-media-genotypes interaction for callus induction in different *T. alexandrinum* genotypes (old seedling stage)

Genotype - Media interaction (callus induction %)

	A	B	C	D	
FAO-1	24.17	48.34	53.34	40.83	41.67
JHB 146	36.67	35.84	22.50	10.00	26.25
BL 142	38.33	37.50	28.33	4.17	27.08
3-90 H	62.50	54.17	55.84	20.00	48.13
9-90 N	12.50	4.17	22.50	4.17	10.83
1-90 P	3.33	37.50	42.50	0.00	20.83
Mean	29.58	36.25	37.50	13.19	29.13

Genotype - Media interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
FAO-1	70.84	45.00	34.17	16.67	41.67
JHB 146	36.67	55.00	13.33	0.00	26.25
BL 142	0.00	36.67	63.33	8.33	27.08
3-90 H	62.50	71.67	58.34	0.00	48.13
9-90 N	12.50	12.50	18.34	0.00	10.83
1-90 P	3.33	0.00	37.50	42.50	20.83
Mean	30.97	36.81	37.50	11.25	29.13

Genotype - Media interaction (callus induction %)

	Collar	Hypocotyl	Petiole	Leaf	Mean
A	46.11	38.33	28.33	5.56	29.58
B	32.22	40.00	51.67	21.11	36.25
C	28.89	35.56	67.22	18.33	37.50
D	16.67	33.33	2.78	0.00	13.19
Mean	30.97	36.81	37.50	11.25	29.13

ANOVA table for old explants of different *T. alexandrinum* genotypes

source	df	ss	ms	F
a	5	14945.562	2989.112	4.577**
b	3	8997.748	2999.249	4.592**
c	3	10849.716	3616.572	5.538**
axb	15	9149.120	609.941	0.934
axc	15	31842.041	2122.803	3.250**
bxc	9	9879.113	1097.679	1.681
axbxc	45	29388.780	653.084	
total	95			

a= Genotype   b= media   c=explant

** = significant at 1% level   160

physical gradient is present within the callus mass (Hall,1991). Such heterogeneity may be the main reason for the differences observed in response of various calli on sub-culture to the differentiating media..

The frequency of shoot initiation depends upon the physiological age of the explants, the older the tissue, the lower the frequency of shoot production (Ayyappan and Rajkumar, 1988). The importance of age of explants in determining the morphogenetic expression of pea leaflet has been demonstrated (Moraginski and Kartha, 1981).

The study therefore showed that the callus induction and subsequent differentiation to root and shoot depended on the interaction of various factors. No uniform protocol could be followed in different species of a genus or even in different genotypes of a single species. A wide spectrum of media, genotype, explant source and explant age were used in the present study and their interactive effect have given successful regeneration in different genotypes and species of *Trifolium*, which will be very useful in future biotechnological efforts in genetic improvement programme of this genus.

5.3. Characterization of calli and regenerant at various stages of growth :

Plant tissue culture *per se* has long been considered as a rich and novel source of genetic variation. Development of somaclonal variants through callus phase is a cheaper source as compared to other biotechnological tools such as somatic hybridization, genetic transformation, DNA recombination etc. This method can be exploited in species having limited genetic diversity provided a suitable regeneration protocol is developed. Reports are available about generation of somaclonal variation and its characterization in different crops such as sugarcane (Heinz *et al.*, 1977), potato (Shephard *et al.*, 1980).

Various reasons have been given for the origin of such variations. The details are described in part 2 (Review of Literature) of this thesis. Variation in isozyme profile and chromosomal number has also been implicated as reasons for the origin of somaclonal variants.

5.3.1. Morphological characterization :

In the present programme morphological characterization of regenerant of *T. resupinatum* and *T. alexandrinum* were carried out.

The regenerant plants after their transfer to field were compared with the mother plant. The metric traits such as plant height, leaf length and breadth, branch number etc. could not be recorded as there was variation in age and stage of plants. Thus the parameters such as branching pattern, flowering initiation, seed set, stem colour etc. were recorded in mother plant and regenerant in *T. alexandrinum* and *T. resupinatum*. The study indicate the production of somaclonal variants which show diversity for these traits. The characters such as flowering initiation, branching pattern showed marked difference from the mother plant.

Previous reports indicate that the somaclonal variation is derived either from the release of genetic diversity pre-existing in the explants or else from the variability originating during cell de-differentiation or callus maintenance *in vitro* ('D'Amato, 1985, 1990). It has been reported to be dependent on the plant species, the genotypes, the type of explant and culture media (Meins, 1983, Karp and Bright, 1985)

5.3.2. Biochemical characterization of mother plant, regenerant and calli of different ages :

In the present programme, the calli at different stages of *in vitro* culture and regenerants were compared with mother plant using zymogram technique (Hunter and Markert, 1957) of five isozymic pattern. The study was carried out in *T. resupinatum*, one genotype each of diploid and tetraploid *T. alexandrinum*.

Isozymes are multiple molecular forms of an enzyme with similar or identical substrate specificities occurring in the same organism (Markert and Moller, 1959). Thus, the variation in isozyme profiles indicate that the variations observed in regenerant has genetical basis and such regenerant can be exploited in future genetic improvement programme.

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In the present study, regenerant of *T. resupinatum* showed wide variation as compared to the mother plant and some other accessions of the same species. The study revealed that the regenerant differs significantly from the mother plant. While no variation was recorded between mother plant and regenerant for esterase and acid phosphatase isozyme profiles, the two plants differed widely for other isozymes (Fig. 5.13).

Isozyme profile of Glutamte Oxalo acetate Transaminase which is a very important enzyme for transfer of amine groups has shown wide variation. The mother plant indicate the presence of 5 bands whereas in the regenerant only band numbers 3 and 4 were recorded. It indicates that the first two bands with high molecular weight and low mobility and the last band with high mobility and low molecular weight are absent in regenerant. Isozyme pattern of SOD in regenerant also indicate absence of band number 2 out of total 3 bands present in the mother plant.

Peroxidase isozyme which is involved probably in IAA metabolism, is a key enzyme in the growth and differentiation process of *in vitro* culture (Scandalios and Sorenson,1977). Wide variation for cathodal bands of this enzyme was observed in mother and regenerant. In fact, a slow migrating band was observed in the regenerant which was not present in mother plant as well as other accessions of this species.

Variation in esterase and acid phosphatase was reported in tobacco callus culture (Bassiri and Carlson, 1979), in wheat and barley calli during differentiation (Chawla, 1988), in Guinea grass between embryogenic and non-embryogenic calli (Alarmelu *et al.*, 1999). Novel acid phosphatase bands during cytodifferentiation in callus cultures of *Vigna* were reported by De and Roy (1984). In this study also novel bands of peroxidase were found.

In another study non-morphogenetic calli of different ages and the mother plant and regenerant in two genotypes (one diploid and one tetraploid) of *T. alexandrinum* were compared for four isozyme profiles. The results indicate that while no variation was detected for esterase, ACP and SOD in the diploid genotype JHB 146, the peroxidase banding pattern for both anodal and cathodal bands

Fig. 5.13 : Isozyme profile in mother plant and regenerant of *T. resupinatum*

Acid phosphatase

Mother Regenerant

Super oxide desmutase

Mother Regenerant

Glutamate oxalo
Transaminase
Mother Regenerant

Esterase

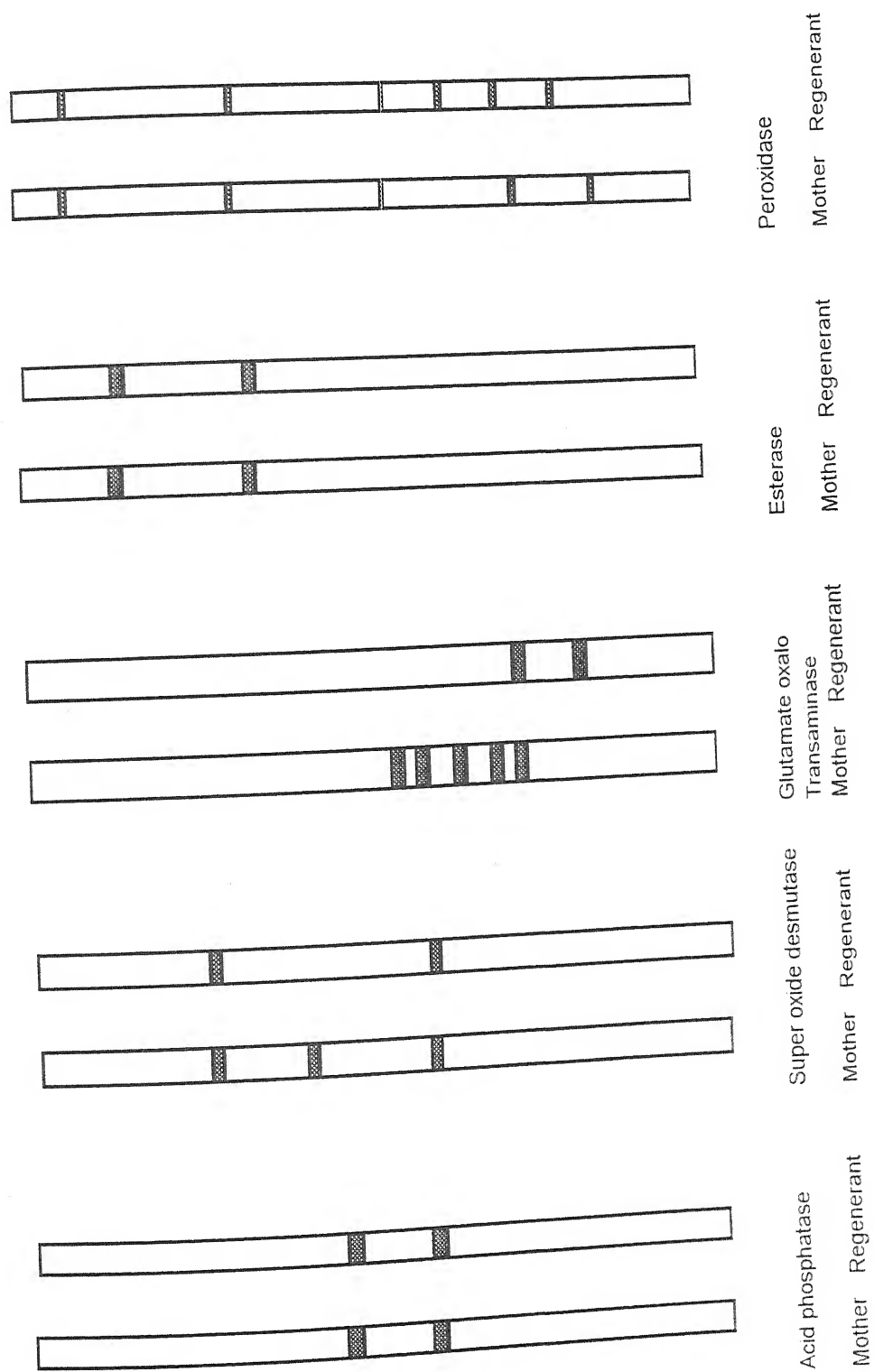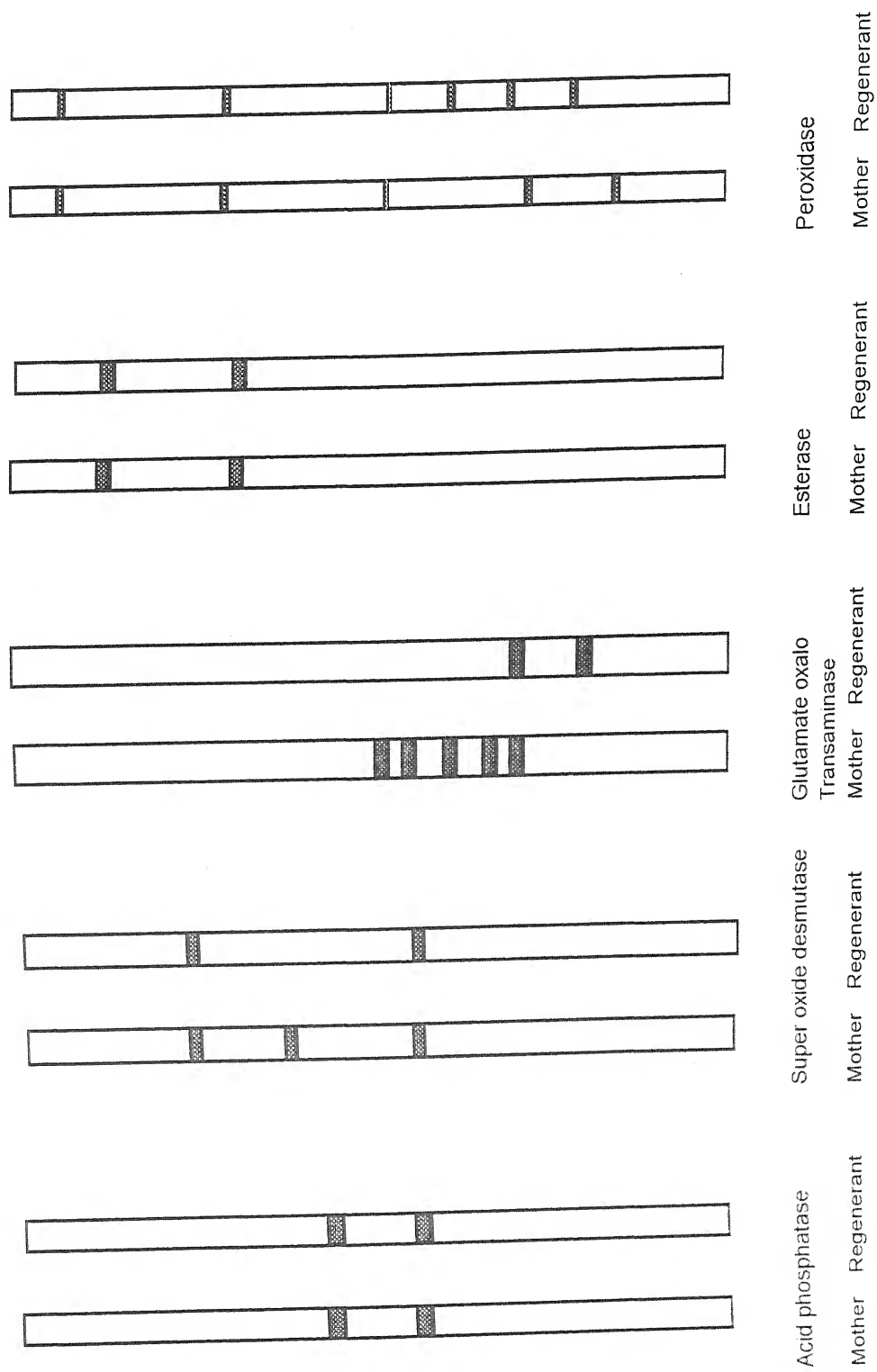Mother Regenerant

Peroxidase

Mother Regenerant

Fig. 5.13 : Isozyme profile in mother plant and regenerant of *T. resupinatum*

showed wide variation. In the mother plant, 5 cathodal bands of peroxidase were present whereas in calli of different ages as well as the regenerant both at vegetative and flowering stage showed less number of bands. The banding pattern for anodal bands indicate that novel bands have appeared during the process of differentiation. While mother plants showed three bands (band no.3.5,7), calli at different stages showed presence of two new high molecular bands with low mobility (Fig.5.14).

In the tetraploid genotype, 1-90 P, SOD and ACP banding pattern showed no variation. One extra band for esterase with low molecular weight and high mobility was noticed in 100 days old non – morphogenetic calli (Fig.5.15). The variation recorded in this tetraploid genotype for anodal and cathodal bands of peroxidase also followed a more or less similar pattern as that of diploid genotype.

Variation in isozyme pattern has been indicated in the differentiation process. Study of acid phosphatase isozymes in *Panicum maximum* (Alarmelu *et al.*, 1999) and in many grasses (Lorenc-kubis and Morawiecka, 1985) and of esterases in guinea grass (Alarmelu *et al.*, 1999 and maize (Everett *et al.*, 1985) have been suggested to be of use as markers in embryogenic and non-embryogenic calli. Variation in glutamate dehydrogenase was reported at various stages of callus growth in *Phaseolus vulgaris* (Arnison and Boll, 1974). They reported change from a pattern of five to a single electrophoretic band after subculture and gradual return to five at end of the culture period.

Isozyme analysis has been used to explain the genetic basis of certain somaclonal variations. If the variation is caused by a loss of a large portion of a chromosome or it involves the alteration or removal of a large number of genes, it is expected that it will result in loss or addition of isozyme bands relative to standard phenotype. In contrast, if only a single gene is altered, it may not reflect in isozyme pattern.

Plant cell growth *in vitro* has been reported to exhibit cytological and isozymic variations, which do occur due to culture conditions. Such variations have been documented in review by Larkin and Scowcroft (1981). The structural changes and numerical variation in chromosomes of cultivated cell and further

Fig 5.14: Zymogram for different isozymes in mother plant, regenerant and calli of different ages in *T.alexandrinum* (JHB 146)
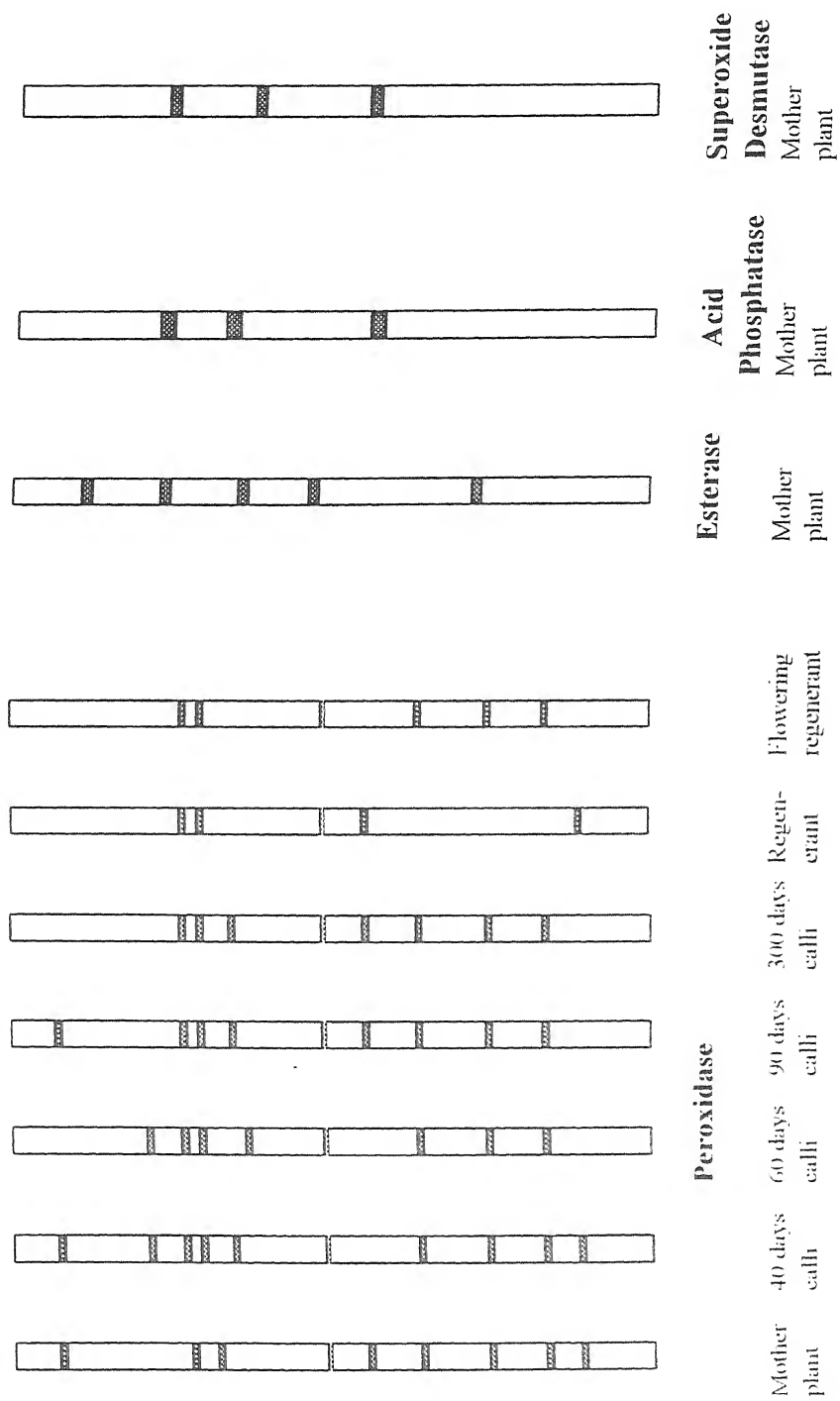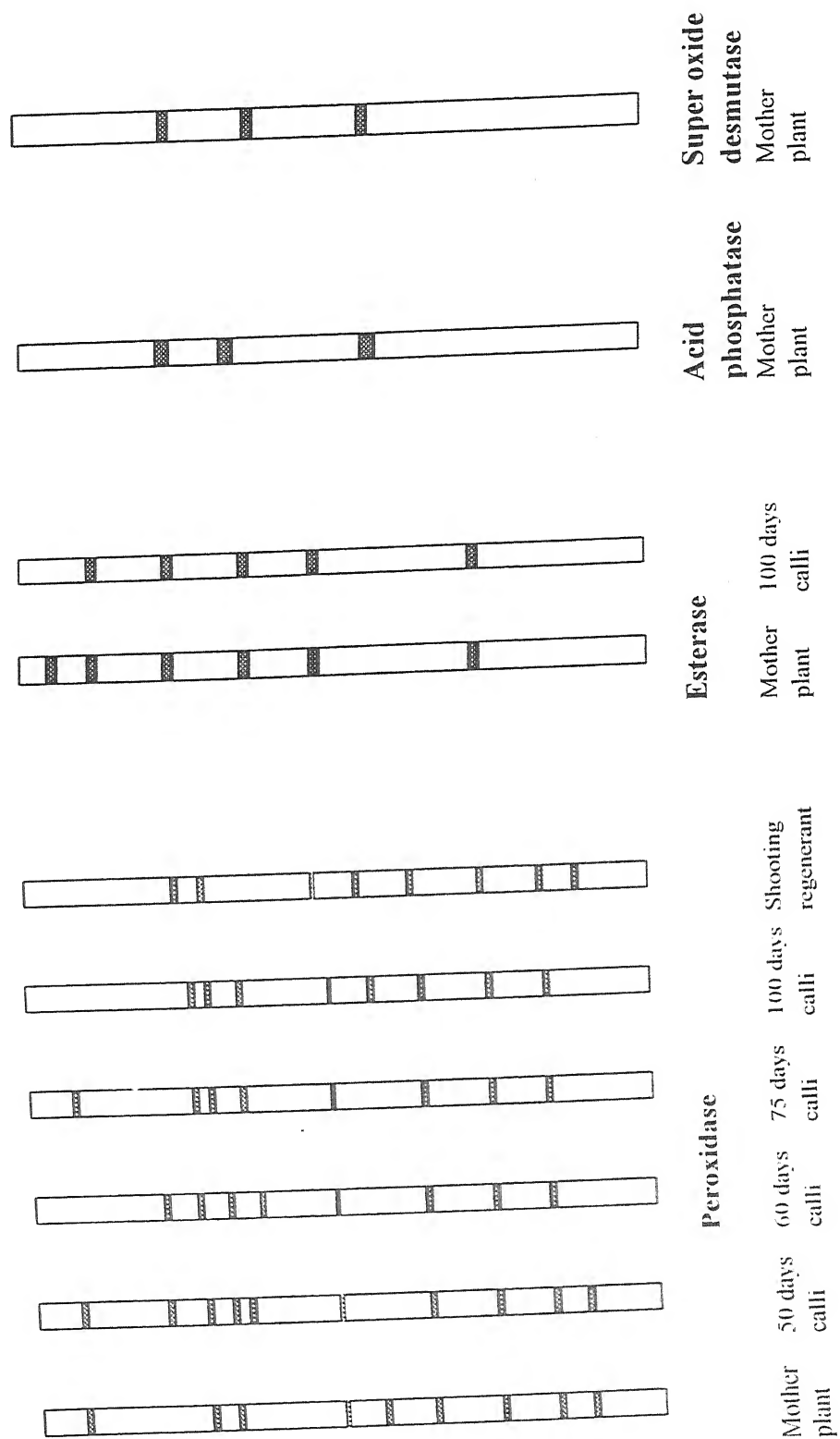
Fig 5.15: Zymogram for different isozymes in mother plant, regnerant and calli of different ages in *T.alexandrinum* (1-90P)
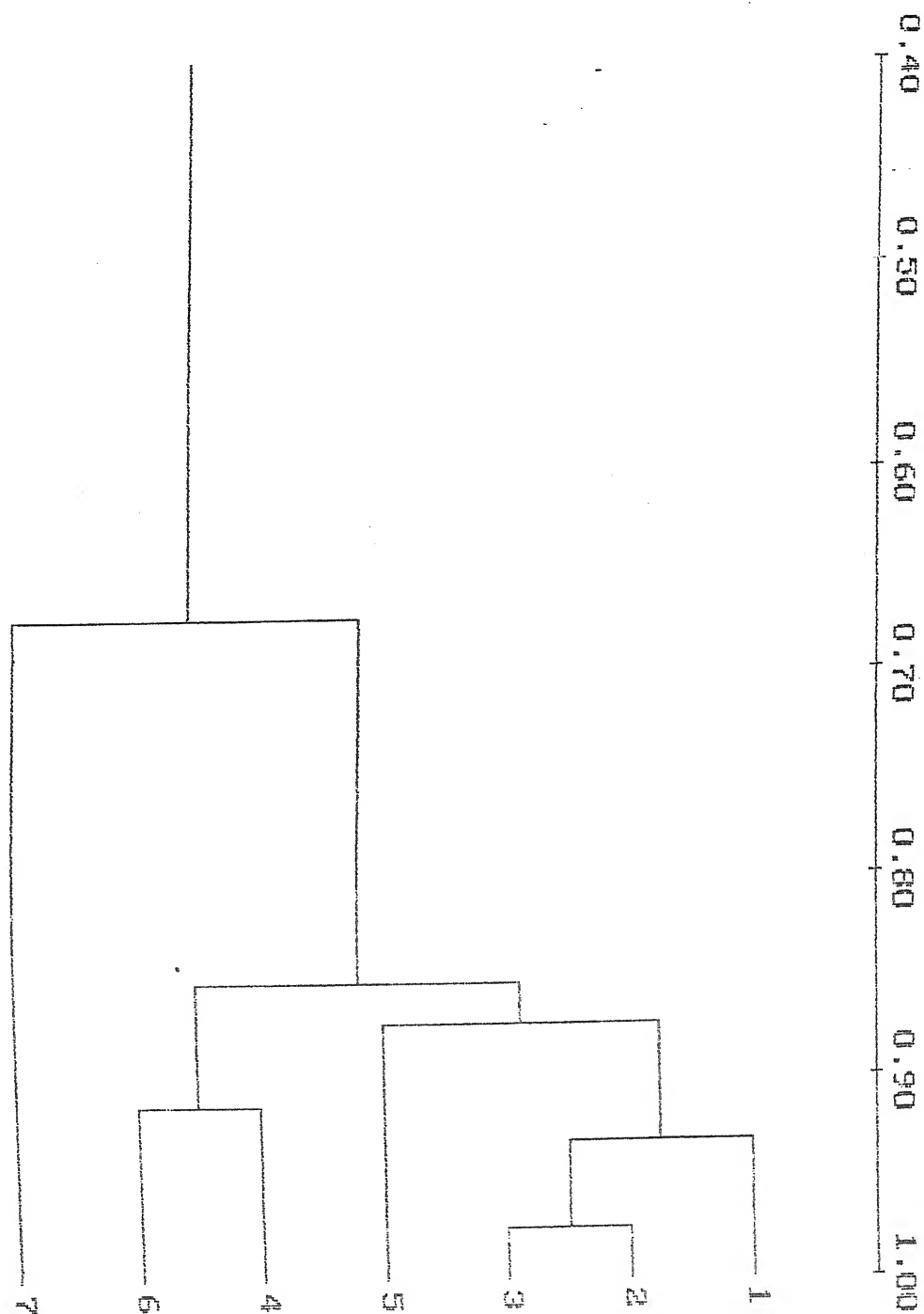
Fig. 5.16 . Dendrogram based on cluster analysis of isozyme data
showing genetic relatedness between accessions, regenerant
and mother plant of *T. resupinatum*

Accessions

1. SH 98-34,   2. SH 98-34-1,   3. SH 98-15,   4. SH 97-BS-1
5. SH 97-BS-2,   6. SH 98-49 (mother plant),   7.   SH 98-49 (Regenerant)

regeneration of plants from these cells may give rise to somaclonal variant which sometimes is of importance in crop improvement programme.

In the tetraploid genotype, it was observed that both the mother plant and regenerant share common banding pattern for SOD, ACP, Esterase and cathodal peroxidase, whereas one low molecular band of anodal peroxidase was absent in the regenerant. In the diploid genotype JHB 146 the two regenerants at different stages showed variation for both anodal and cathodal bands of peroxidase which includes presence of novel bands.

Presence of some novel bands such as band 6 of Esterase and band no. 1, 2, 4, 6 of cathodal peroxidase indicate formation of new genetic combinations or triggering of some new genes in the non-morphogenetic calli during the culture process resulting in the production of new gene products.

5.3.3. Cytological variation during *in-vitro* culture stages of calli in *T. alexandrinum*

Cytological study of the diploid and tetraploid lines of *T. alexandrinum* was carried out at different stages of callus growth in one diploid genotype JHB 146 and one tetraploid genotype 1-90P. The calli were induced from hypocotyl explant on 'A' media and were maintained on 'A' media. The calli were non-morphogenetic and did not show any differentiation during this process. Regular sub-culturing was done at 30 days interval in the same media which resulted in only callus proliferation.

In the present study involving two genotypes, a common trend observed in both cases was that with the advancing age of the calli the number of polyploid cells increased. The calli of diploid genotype showed only 36.8 % diploid cells in 300 days old calli against 65.6% tetraploid cells in calli derived from the tetraploid genotype which shows that increase in somatic chromosomes number beyond tetraploid level is less preferred in callus cells. The cells with higher ploidy level showed clumping of chromosomes and in some cases laggards were also observed at anaphase. Higher frequency of polyploid cells in older calli has also been reported by Mathur and Prakash (2000) in *Vigna mungo*. Formation of polyploid

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cells can be attributed to endomitosis which might have occurred due to interaction between growing explants tissue with culture condition including temperature, nutrients and the concentration of hormones. Restitution nuclei formation due to the spindle failure and chromosome lagging was also reported to be cause of polyploidization in *in vitro* cultures (Bayliss, 1973). Szeykowaka (1974) has reported that efficiency of 2, 4-'D' improves in association with kinetin in DNA synthesis and mitosis.

As regards regeneration or shooting from the calli of different ages, it was observed that with advancing age, the calli were not responding well to the shooting media. Multiple shoot emergence was observed in the calli of 30 to 35 days age. Poor response of old calli for shooting was attributed to the higher frequency of polyploid cells. This phenomenon was observed in contrast to the earlier report of Mathur and Prakash (2000) in *Vigna mungo* who have reported no apparent relationship between the ability of a callus to regenerate and the frequency of diploid, polyploid or aneuploid cells.

The concomitant occurrence of the loss of morphogenetic potentiality and a high degree of nuclear irregularity and a definite effect of the degree of irregularity on the differentiation indicate some relationship between these nuclear aberrations and the organogenesis *in vitro*. Torrey (1967) and Singh (1986) have reported that such changes in cells disturbs the physiological and genetical balance of the callus leading to loss of the capacity of the regeneration.

These chromosomal aberration together with the variation in isozymic profile of non-morphogenetic calli carried out in two *T. alexandrinum* genotypes indicate that these calli have accumulated genetic variations/mutations which has affected their totipotency and regeneration capability. Both the genotypes although differing for their ploidy level indicate a similar trend.

SUMMARY

6. SUMMARY

The genus *Trifolium,* commonly called clovers, comprises of 237-300 species. Twenty five species of the genus are important as cultivated forages and pasture crops for both wild and domestic animals. The genus includes both annual and perennial types and is distributed over temperate to sub-temperate and tropical to sub-tropical climate. The center of diversity of the genus *Trifolium* L. (family Leguminosae) lies in the Mediterranean belt and later waves of colonization spread the genus to the Americas and Africa.

The important perennial pasture species of the genus are *T. repens* (White clover), *T. pratense* (Red clover), *T. hybridum* (alsike clover), *T. ambiguum* (caucasian clover). These species form the backbone of the livestock industry in temperate part of the globe. The most common annual species are *T. alexandrinum* (Egyptian clover or Berseem), *T. resupinatum* (Persian clover or shaftal) and *T. subterranean* (subterranean clover). These species are cultivated as winter annuals in the tropical and subtropical regions.

T. alexandrinum commonly called as Egyptian clover or Berseem is believed to have originated in Egypt and / or other Mediterranean countries. Its center of diversity lies in Mediterranean zone such as Egypt and Turkey. Berseem cultivar Mescavi or Meskawi was introduced in India in the year 1903. It is widely adapted as a cultivated, nutritive green fodder for the dairy animals and is cultivated in about 2 million ha area with an average national productivity of 85 t /ha green fodder. Certain features like multicut nature (4-6 cuts/season), long duration of green fodder availability (November to April), high yield (85 t/ha), good quality (20% crude protein), good digestibility (70% IVDMD) and palatability have made it highly acceptable among farmers.

Genetic improvement programme carried out through conventional genetical and breeding techniques at various research institutes and universities in India and abroad have not resulted in any significant outcome. The efforts have reached at a road block where biotechnological efforts need to be considered for a breakthrough. The main obstacle faced is narrow genetic base of the crop. Decades

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of selection for high biomass production has robbed the crop of its genetic diversity.

The plausible approach to broaden the genetic base of Berseem is therefore, through different biotechnological techniques such as *in vitro* regeneration, somaclonal variation, somatic embryogenesis, genetic transformation *etc.* Transfer of alien genes from wild taxa through somatic hybridization together with *in vitro* embryo rescue following interspecific hybridization may help in broadening the genetic base.

Plant tissue culture technique has provided potentially powerful new tools for generating, selecting and propagating novel and economically important plant varieties. Plant tissue culture technique has been developed and refined for many economically important plants during the last few decades. It exploits the theory of totipotency, which is probably a characteristic of all living plant cells.

The primary requirement for many biotechnological approach to genetic improvement is to develop suitable protocol for *in vitro* regeneration and successful establishment of regenerants in field conditions. The characterization of regenerants at various stages of development will give a picture of the variability.

Different species and genotypes may have different nutritional requirements for *in vitro* development, the study therefore should be planned to work out the efficacy of different explants, genotypes and media. The protocol developed for *in vitro* regeneration in different species can be exploited in the long run for various biotechnological tools such as somatic hybridization, somaclonal variation, genetic transformations *etc.*

The proposed programme was therefore carried out with the long term objective of creation of genetic diversity in different *Trifolium* sp. particularly *T. alexandrinum*. In the present study the efforts were made

- to develop suitable protocol for *in vitro* regeneration in genus *Trifolium* with particular reference to *T. alexandrinum*.
- to induce genetic variability through somaclonal variation
- to characterize callus/regenerant at various stages of growth.

The present study involved six genotypes of *T. alexandrinum* and 6 species of *Trifolium*. The genotypes and species were selected so as to get a range of wide genotypic response towards *in vitro* culture. The species selected ranged from temperate species to sub-tropical one.

A wide range of different media combinations with varying level of growth hormones were tried for callus induction and organogenesis as well as somatic embryogenesis. The media used incorporated different ratios of auxin and cytokinin so as to get a better regeneration. A total of six explants *viz*. root, collar, cotyledon, hypocotyl, petiole, leaf of different genotypes and species of *Trifolium* were tried to see their interaction with media.

Since various reports are available which indicate that age of seedling from where explants are taken affect the *in vitro* response, 10 days and 30 days old seedling were selected and explants were taken from these two sources in six genotypes of *T. alexandrinum* to ascertain the effects of age of explants.

In this study the regnerants and non-morphogenetic calli at different stages were characterized by morphological, biochemical and cytological parameters. The qualitative traits such as flowering pattern, seed set, colour of stems and leaves, branching pattern were compared in mother plant and regenerant. The biochemical studies include isozymic banding pattern of five isozyme systems *i.e.* peroxidase, esterase, acid phosphatase, super oxide desmutase, and Glutamate oxalo acetate transferase. The regenerant and calli at different stages were characterized and statistical analysis was also carried out. The regenerated plants were found to vary from the mother plants and they are termed as somaclonal variation.

The present study was carried out to see the interaction of different factors such as genotype, media growth regulators combinations, explants source, age of explants etc. towards *in vitro* response.

The experimental findings indicate differential response of various explants in different species. The response towards callus induction frequency, nature and growth rate of callus, its response towards organogenesis and differentiation varied widely in different explant-media-genotype combinations.

Media– explant source interaction response in six *Trifolium* species

T. resupinatum : Out of the two media selected, one was without auxin ('A-1') and the other comprised of both auxin and cytokinin ('A'). Results indicate that the media without auxin although produced calli but the calli were non-morphogenetic and did not show any organogenesis on sub-culturing . Good organogenetic potential was noticed in the calli developed from cotyledon, hypocotyl and root in 'A' media where shoot formation was noticed. These shoots also produced roots when transferred to 'RL' medium. Thus, the regeneration protocol was developed for this species.

In further studies involving five other species of *Trifolium* two media 'A' and 'D' were used. Auxin and cytokinin level varied in these two media formulations. The ratio of auxin to cytokinin was kept as 0.5:1 in media 'A' and 5:1 in media 'D' to study the effect of their quantitative level as well as their interactive role. Six explants were used in all five species to see their *in vitro* response.

T. subterraneum : *In vitro* interaction response in genotype IG 96-112 of *T. subterraneum* indicated a media-explant dependent effect on callus induction and regeneration. The high hormone media 'D' showed better callogenic properties as compared to low hormone media 'A' for all the six explants studied.

Very poor callus inducing response of 'A' media was observed for leaf, petiole, cotyledon, hypocotyl explants, while collar and root explants showed moderate response as 50% of the explants showed successful callus induction. The study indicates that for successful callus induction high amount of auxins and cytokinins along with high auxin : cytokinin ratio is required.

Nature of callus formed was more dependent on explants than the media combination. Calli obtained from leaf, cotyledon and collar were compact in nature, whereas those obtained from root were nodular in both the media. Similarly calli induced from hypocotyl and petiole were largely friable in nature.

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The friable green calli obtained from 'D' media-hypocotyl combination showed induction and multiplication of shoots in 'E' media and root formation in RL media. This combination thus resulted in a successful protocol for regeneration in this genotype of *T. subterraneum*.

Embryogenic response of calli obtained from various combinations were also poor. Green, globular embryo like structures were observed in some cases but these did not develop into plantlets.

Earlier workers have also used various media combinations for study in this species. No morphogenetic response was observed in MS supplemented with Nicotinic acid and Kinetin. The basal media used in the present study is L2. Using the same basal media supplemented with different levels of hormones, regeneration *via* organogenesis and embryogenesis have been reported.

Trifolium repens : Plant regeneration in *T. repens* has been reported by many authors using different levels of hormones and basal media. Genotypic differences have been attributed to the variation in results obtained from time to time.

In the present study on *T. repens* EC 400986, callogenic potential of explants such as root and collar was found to be very poor, whereas that of petiole was very good irrespective of media. High hormone media 'D' was more effective for cotyledon and leaf explants. Friable calli were obtained in high frequency only from petiole explants.

As regards shoot and root induction from the calli, petiole explants derived calli from both the media showed induction of shoots in equal frequency. However, root induction could be obtained only in shoots derived from calli induced in 'D' media. 40% plants could be transferred to field.

Prolific plant regeneration was reported by previous authors on MS media containing NAA and BAP, while other phytohormone combinations, 2, 4-D or picloram with kinetin 2-ip resulted in either extensive callus formation or distorted shoot development. Much difference has not been reported in frequency of shoot formation in eight cultivars of *T. repens*. Our results are in confirmation of earlier

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results that NAA and BAP are good for regeneration in *T. repens* and its concentration during callusing from petiole has no bearing on shoot induction.

T. apertum : *T. apertum* is a diploid species domesticated in Russia and possesses close phenotypic resemblance to *T. alexandrinum*. This species has not been included in any tissue culture study earlier but due to its close affinity with *T. alexandrinum* it was included in present study.

Low hormone media 'A' failed to show callus induction in 5 out of 6 explants of genotype EC 401712. Only in hypocotyl very low frequency of compact and green calli was observed.

High hormone media 'D' showed good callogenesis in hypocotyl, cotyledon and petiole explants. In collar, leaf and root explants the frequency of callus induction ranged from 0 to 14%. Friable green calli were observed in hypocotyl and petiole explants which also showed medium growth rate. The calli from petiole explant –'D' media combination showed very good organogenetic and embryogenetic potential on sub-culture. 50% of sub-cultured tubes showed shoot induction and a large number of them also showed root induction in rooting media. A suitable protocol for regeneration and transfer of plantlets *in vitro* in this genotype of *T. apertum* has been developed probably for first time.

Trifolium glomeratum : The *in vitro* study in genotype EC 401700 of *T. glomeratum* showed no response of root and collar explants to callus induction whereas in other media–explant combinations, good callus induction was observed. Media 'A' was more effective for hypocotyl explant whereas cotyledon and petiole explant were more responsive to media 'D' so far as callus inducing efficiency is concerned. Friable, green calli could be obtained only in petiole –'D' media combination which also showed good organogenetic potential. The plantlets could be recovered and transferred to the field by successful root and shoot induction in these calli. The calli obtained from other combinations showed only callus proliferation and no organogenesis was observed.

Trifolium hybridum : The genotype EC 401702 showed very good frequency of callus induction in all the combinations except for hypocotyl, and cotyledon

explants in 'A' media. Callus induction was excellent in leaf, collar, petiole, root in both the media combinations. Nature of the calli obtained was very much dependent on explant source as the leaf, cotyledon and collar showed compact calli whereas friable, green calli with better growth rate was found in hypocotyl and petiole explants which showed organogenesis after subculturing in shoot inducing media. Successful regeneration could be obtained in hypocotyl induced calli on both 'A' and 'D' media. Petiole induced calli on media 'A' resulted in shoot formation and multiplication in 'E' media but rooting could not be induced. Although leaf and collar responded to callus formation in both the media but no differentiation could be observed on sub-culture, as most of the calli turned brown after sub-culturing.

Previous studies do not report regeneration protocol in this species. Callus growth was obtained from seedlings of Alsike clover cultured on SH medium supplemented with 2, 4-D, CPA and KIN in different concentrations, however, no morphogenetic development was reported by earlier workers. Hence, this seems to be the first successful regeneration of complete plantlet of this species.

Media– explant source interaction response in six *Trifolium* species

In all the 6 species studied, an apparent genotype, media explant response was observed. The callus induction frequency, nature of callus and their growth rate varied in different combinations. Summarizing the observations of 5 species where same media and explant combinations were used, some interesting observations emerged. Species such as *T. hybridum* showed better response towards callus induction (72.1%) followed by *T. subterraneum* (48.7%). High hormone media 'D' was found to be far superior (54.4%)than low hormone media 'A' (23.9%) for callus induction. Media 'D' was more effective in all the six explant media combinations. Similarly, the callus induction response was very high in leaf, petiole, cotyledon and hypocotyl explants. The differential response of explants of different species to varying hormonal concentration can be attributed to the nature of explant tissue, its genetic potential for regeneration and hence, its nutritional requirement. It has been observed that nutritional requirements for optimal growth of a tissue *in vitro* vary with the species or with the genotypes

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within the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth.

Response of root and collar explants were generally very poor except in *T. subterraneum* and *T. hybridum*. This may be due to the fact that the first species is prostrate with profuse growth and the later species is a perennial and more persistent, thus, have more active growing roots. It seems that in these two species roots have more active meristematic zones and so their response to callogenesis was better. However, the calli did not show any regeneration potential even after repeated sub-culturing. In general, response of petiole explants was best among the six explants studied.

It was observed that only friable, green calli were responsive to organogenesis in shoot inducing media (media 'E'). Successful shoot induction was observed only in calli derived from following combination :

T. resupinatum : Hypocotyl - 'A', Cotyledon- 'A', Root- 'A' media.

T. subterraneum : Hypocotyl - 'D' media

T. hybridum : Petiole -'D' , Hypocotyl – 'D' and 'A' media

T. glomeratum : Petiole - 'D' media

T. apertum : Petiole -'D' media

T. repens : Petiole -'D' and 'A' media

The root inducing media 'RL' was found to be good for all the species. Satisfactory results were obtained in inducing good quality roots in almost half of the shoots. In this study only one media was tried and more efforts were put towards good quality callus and shoot induction, which seemed to be a problem in these species as per earlier reports.

In all the genotypes petiole and hypocotyl in 'D' media was found to be the best combinations for morphogenetic callus induction. The study resulted in development of protocol for regeneration of plants in all six species included in the present study.

In vitro response in *Trifolium alexandrinum* L.

The present study included six genotypes (3 diploid, 3 tetraploid), four explant sources, two explant ages, 4 media formulations each for callus induction and shoot induction in different combinations. The findings are summarized as follows.

Effect of seedling age of explants

The study was carried out with the assumption that the different explants will vary in their developmental stage when excised from 10 and 30 days old seedlings in different diploid and tetraploid genotypes. The results indicate variable response towards callusing in six genotypes under study with respect to the age of the seedling and explant sources and media.

Diploid genotypes : Response of individual genotypes revealed that there was no marked difference for young and old explants in the genotype FAO-1 for callus induction frequency. Old collar explants showed better callus induction whereas other explants of young age were more responsive. Response of old explants was slightly better in high hormone media ('C' and 'D'). Young explants of JHB 146 showed better (32.92%) response than old explants (26.25%), although it was not statistically significant. Old leaf explants did not respond to callus induction in any media. This may be due to the fact that old leaves may have more permanent cells and less meristematic tissue. Old explants of collar and hypocotyl showed better response than younger ones, while the reverse was true for slow growing part such as petiole. The third diploid genotype BL 142 showed better response of young explants for callus induction frequency than the older explants of collar, hypocotyl and leaf. Old petiole explants were more responsive than younger ones. High hormone media 'C' and 'D' induced calli in higher number in young explants whereas in low hormone media ('A' and 'B') the callus induction response was similar in both the ages.

Tetraploid genotypes : It seems that the nature of explants has a clear effect on the callogenic potential of two explant ages as observed in tetraploid genotype 3-90 H. While the young meristematic zone derived explants such as collar and

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hypocotyl showed high callus induction in old age, there was little difference in petiole and reverse was true for leaf. Leaf explants when fully mature showed no callogenic response while younger leaves showed good percentage of callus induction. High hormone media 'D' was more responsive in young explants whereas low hormone level showed better response for older explants. In general, older explants showed better response (48.1%) as compared to young explants (33.96%).

The response of genotype 9-90N also showed marked difference for callogenesis with respect to age of the explant. The old explants showed poor response in all the media and explants source whereas the young explant were better responsive. Young explants of the third tetraploid genotype 1-90 P were superior (46.79%) than the older explants (20.83%) for callus induction when data of all the four explant source was pooled. Older explants showed very poor response at two extremes of hormonal level ('E' and 'D'), and good response in media 'B' and 'C' which has intermediate level of growth regulators. Leaf and petiole explants showed better response in older explants than the collar and hypocotyl.

Thus, the age of various explant was found to be important factor affecting the callusing response in different media. Earlier workers have also reported that the explants of young tissues, generally form callus which undergo cell division more rapidly than older tissues. In Vitis, the age of explant was found to be very important for development of organogenic calli and the younger leaves were most efficient. Physiological maturity and segmental age of the explant was found to play a vital role in the callogenic response of the explant in Indigofera wherein response of seedling explants was found to be better than leaf cultures.

Genotypic response

Many workers have reported that genotypic response varies with respect to culture media. Further, in an out-breeding species like T. alexandrinum, T. repens it has been reported that strains are highly heterogeneous. They show differential response to the culture media and explant combinations. Hence, in the present

study six genotypes represented by two ploidy levels were selected.

Three diploid genotypes FAO-1, JHB 146 and BL 142 belonging to Mescavi group were selected for this study. The callogenic response was more or less similar in the three genotypes with JHB 146 showing a little poor response. Media 'B' was slightly better than other three media. Non-significant difference was observed for callus induction frequency in different media. Similarly, no significant difference was observed in efficacy of different media combinations for callus induction from explants taken from the old seedling. The intermediate hormone level media ('B' and 'C') were more effective than the media at two extremes of low and high hormonal concentrations 'A' and 'D'.

Out of the three tetraploid genotypes, 1-90 P showed best response for callus induction followed by 9-90 N and 3-90 H. Marked difference was observed in the response of three tetraploid genotypes for callus induction when explants were collected from 30 days old seedling. The callus induction ranged from 10.8% in 9-90 N to 48.1% in 3-90 H. Intermediate hormone level media 'B' and 'C' were more effective. Very poor response was seen in high hormonal media for callus induction in tetraploid genotypes.

All four media combinations were found to be equally effective so far as frequency of callus induction was concerned when the data from all the observations were pooled together. As observed in diploid genotypes very good response for callogenesis was observed for hypocotyl explant both in old and young seedling. The old petiole explants also responded well to different media. The young collar and old leaf explants showed very poor response.

Response of different young explants of six genotypes of *T. alexandrinum* to four media combination was also compared and it was observed that hypocotyl was most responsive followed by petiole and leaf. Least response was observed in collar derived explants.

The hypocotyl explants of old seedling was best responsive (55.56%) followed by collar (45%) but leaf and petiole explants showed poor response. Collar explants of old seedling responded better probably because after certain

level of growth the collar region starts growing rapidly.

Effect of explants source :

In the present study four explant sources *i.e.* leaf, petiole, collar and hypocotyl were used. As these explants were excised from six genotypes at two different growth stages and cultured on four media combinations supplemented with varying degree of hormones, the response of different explants was compared in different genotype, media and seedling stage combination.

The explants excised from 30 days old seedling showed differential response to different explants. At old seedling stage, among diploid genotypes, hypocotyl and collar showed better response in comparison to petiole and leaf where callus inducing response was poor. In tetraploid genotypes petiole explants showed best response (38.1%) followed by equal response of hypocotyl (28.1% and collar (26.1%). Leaf showed very poor response in two genotypes and moderate response in third genotype (1-90P).

In diploid genotypes, young hypocotyl explants were found to be far better (57.78%) than other explants source for callus induction capability. Collar showed poor response (22.08%) whereas petiole and leaf showed moderate response with petiole (39.7%) performing better than leaf (30.8%). In tetraploid genotypes also hypocotyl was best among the four explants tried. Leaf and petiole showed equal callogenic capability whereas response of collar derived explants was poor (10.4%).

Media response

As regards the overall response of different media to the four young explants, no marked difference was observed i.e. high or low hormone concentration were equally effective. In diploid genotypes, all the four media were almost equally effective for callus induction. The best response was seen in 'B' media (41.9%) followed by 'C' and 'B'. High hormone media 'D' showed poorest response (32.23%) for callus induction. In tetraploid genotypes also, no major difference was seen among the four media combinations for callus induction

response from different explants.

In diploid genotypes intermediate hormone level media 'B' and 'C' were more effective than the other two media with low and high level of hormones whereas among tetraploid genotypes response of media 'C' was best followed by media 'B' and media 'A'. High hormone media 'D' showed very poor response. In fact, leaf and collar explants of tetraploid genotypes showed no callus induction in media 'D'.

Characterization of calli and regenerant at various stages of growth :

Plant tissue culture *per se* has long been considered as a rich and novel source of genetic variation. Development of somaclonal variants through callus phase is a cheaper source as compared to other biotechnological tools such as somatic hybridization, genetic transformation, DNA recombination etc. This method can be exploited in species having limited genetic diversity provided a suitable regeneration protocol is developed.

In the present programme, the calli at different stages and regenerants were compared with mother plant using morphological and isozymic pattern. Non-morphogenetic calli were also studied cytologically at different stages.

The regenerant plants after their transfer to field were compared with the mother plant. The metric traits such as plant height, leaf length and breadth, branch number etc. could not be recorded as there was variation in age and stage of plants. The parameters such as branching pattern, flowering initiation, seed set, stem colour etc. were recorded in mother plant and regenerant in *T. alexandrinum* and *T. resupinatum*. The study indicate the production of somaclonal variants showing diversity for these traits. The characters such as flowering initiation, branching pattern showed marked difference from the mother plant and indicated the production of novel genotypes.

Previous reports indicate that the somaclonal variation is derived either from the release of pre-existing genetic diversity in the explants or else from the

variability originating during cell de-differentiation or callus maintenance *in vitro*. It has been reported to be dependent on the plant species, the genotypes, the type of explant, culture, media formulations etc.

Isozymic variation was also studied at different stages of *in-vitro* culture process using five isozyme profiles following zymogram technique. Since, isozymes are multiple molecular forms of an enzyme with similar or identical substrate specificities occurring in the same organism, the variation in isozyme profiles have indicate that the variations observed in regenerant has genetical basis and such regenerant can be exploited in improvement programme.

In the present study, regenerants of *T. resupinatum* showed wide variation as compared to the mother plant and some other accessions of the same species. While no variation was recorded between mother plant and regenerant for esterase and acid phosphatase isozyme profiles, the two plants differed widely for other isozymes.

Isozyme profile of Glutamte Oxalo acetate Transaminase which is a very important enzyme for transfer of amine groups has shown wide variation. The mother plant show the presence of 5 bands whereas in the regenerant only band numbers 3 and 4 were recorded. It indicates that the first two bands with high molecular weight and low mobility and the last band with high mobility and low molecular weight are absent in regenerant. Isozyme pattern of SOD in regenerant also indicate absence of band number 2 out of total 3 bands present in the mother plant.

Peroxidase isozyme which is involved probably in IAA metabolism, is a key enzyme in the growth and differentiation process of *in-vitro* culture. Wide variation for cathodal bands of this enzyme was observed in mother and regenerant. In fact, a slow migrating band was observed in the regenerant which was not present in mother plant as well as other accessions of this species.

Variation in esterase and acid phosphatase was reported in tobacco callus culture, in wheat and barley calli during differentiation, in guinea grass between embryogenic and non-embryogenic calli. Novel acid phosphatase bands during

cytodifferentiation in callus cultures of *Vigna* was also reported. In this study also novel bands of peroxidase were found.

Isozyme profile of non-morphogenetic calli of different ages and the mother plant and regenerant in two genotypes (one diploid and one tetraploid) of *T. alexandrinum* showed no variation for esterase, ACP and SOD in the diploid genotype JHB 146, the peroxidase banding pattern for both anodal and cathodal bands showed wide variation. In the mother plant, 5 cathodal bands of peroxidase were present whereas in calli of different ages as well as the regenerant both at vegetative and flowering stage showed less number of bands. The banding pattern for anodal bands indicate that novel bands have appeared during the process of differentiation. While mother plants showed three anodal bands (band no.3,5,7), calli at different stages showed presence of two new high molecular bands with low mobility.

In the tetraploid genotype, 1-90 P, SOD and ACP banding pattern showed no variation. One extra band for esterase with low molecular weight and high mobility was noticed in 100 days old non – morphogenetic calli. The variation recorded in this tetraploid genotype for anodal and cathodal bands of peroxidase also followed a more or less similar pattern as that of diploid genotype.

Previous studies have indicated variation in isozyme pattern in the differentiation process. Isozyme analysis has been used to explain the genetic basis of certain somaclonal variations. If the variation is caused by a loss of a large portion of a chromosome or it involves the alteration or removal of a large number of genes, it is expected that it will result in loss or addition of isozyme bands relative to standard phenotype. In contrast, if only a single gene is altered, it may not reflect in isozyme pattern.

In the tetraploid genotype, it was observed that both the mother plant and regenerant share common banding pattern for SOD, ACP, Esterase and cathodal peroxidase, whereas one low molecular band of anodal peroxidase was absent in the regenerant. In the diploid genotype JHB 146 the two regenerants at different stages showed variation for both anodal and cathodal bands of peroxidase which includes presence of novel bands.

Presence of some novel bands such as band 6 of Esterase and band no. 1, 2, 4, 6 of cathodal peroxidase indicate formation of new genetic combinations or triggering of some new genes in the non-morphogenetic calli during the culture process resulting in the production of new gene products.

Cytological variation during *in-vitro* stages of *T. alexandrinum*

Plant cell growth *in vitro* has been reported to exhibit cytological and isozymic variations, which do occur due to culture conditions. The structural changes and numerical variation in chromosomes of cultivated cell and further regeneration of plants from these cells may give rise to somaclonal variant which sometimes is of importance in crop improvement programme.

Cytological studies were carried out in non-morphogenetic calli of different ages of one diploid and one tetraploid genotype of *T. alexandrinum*.

A common trend between two genotypes observed was that with the advancing age of the calli the number of polyploid cells increased. which shows that increase in somatic chromosomes in callus cells. The cells with higher ploidy level showed clumping of chromosomes and in some cases laggards were also observed at anaphase. Higher frequency of polyploid cells in older calli has also been reported by previous authors. Formation of polyploid cells can be attributed to endomitosis which might have occurred due to interaction between growing explants tissue with culture condition including temperature, nutrients and the concentration of hormones. Restitution nuclei formation due to the spindle failure and chromosome lagging was also reported to be cause of polyploidization in *in vitro* cultures.

As regards regeneration or shooting from the calli of different ages. it was observed that with advancing age, the calli were not responding to the shooting media. Poor response of old calli for shooting was attributed to the higher frequency of polyploid cells. This phenomenon was observed in contrast to the earlier report in *Vigna mungo* where no apparent relationship between the ability of a callus to regenerate and the frequency of diploid. polyploid or aneuploid cells.

The concomitant occurrence of the loss of morphogenetic potentiality and a high degree of nuclear irregularity and a definite effect of the degree of irregularity on the differentiation indicate some relationship between these nuclear aberrations and the organogenesis *in vitro*. It has been reported that such changes in cells disturbs the physiological and genetical balance of the callus leading to loss of the capacity of the regeneration.

These chromosomal aberrations together with the variation in isozymic profile of non-morphogenetic calli carried out in two *T. alexandrinum* genotypes indicate that these calli have accumulated genetic variations/mutations which has affected their totipotency and regeneration capability. Both the genotypes although differing for their ploidy level indicate a similar trend.

The present study has resulted in successful development of regeneration protocol of six *Trifolium* species and four *T. alexandrinum* genotypes. The successful regeneration in *T. glomeratum* and *T. apertum* is probably the first report in these two species. The study has shown that various factors such as age of explant, source of explant, hormonal composition of culture media, genotype play important roles in the *in-vitro* callus induction and subsequent morphogenesis. An interaction of all the above factors determine the callus induction, its nature and subsequent differentiation. The diploid genotypes of *T. alexandrinum* were found to be more responsive than the tetraploid genotypes so far as regeneration is concerned. In the diploid genotypes the regeneration could be obtained mostly from the genotypes which are 30 days old which indicate that the physiological maturity of the explant tissue is important for the differentiation process. The explants which are actively growing such as hypocotyl, petiole were found to be more responsive as compared to the old tissue such as leaf. The high hormone level media was more effective in different species as compared to low hormone media. Presence of auxin in the callus induction media seems to have a positive role in further differentiation of the calli as observed in the study of *T. resupinatum*

The characterization of non-morphogenetic calli revealed that the accumulation of high ploidy cells may be responsible for the loss of differentiating ability of the callus. This was also supported by changes in the isozymic banding

pattern. The regenerant show variation both phenotypically and isozymically which indicate that the process of *in vitro* regeneration has resulted in development of somaclonal variants. Such variants can go a long way in enrichment of narrow genetic base in Berseem.

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